EXHIBIT 14

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Original Paper

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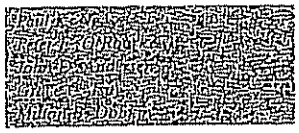

Process-Scale Purification of Immunoglobulin M Concentrate

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Abstract

An IgM concentrate was purified from Cohn fraction III. Efficiency of euglobin precipitation was shown to be controlled by pH and ionic strength. Prekallikrein activator activity in the product was insignificant. Overall yield from the octanoic acid supernate and purity of the concentrate were 66 ± 8 (n = 16) and 50 ± 5% (n = 16), respectively. Solvent-detergent treatment to inactivate lipid-enveloped viruses was demonstrated and implemented into the process. Process studies to control residual virucidal agents and C4a generating activity are presented.

Introduction

The Cohn plasma fractionation process yields a waste product, fraction III [1]. Fraction III is known to be a source of an immunoglobulin fraction enriched in IgM. Octanoic acid was employed to precipitate lipids and lipoproteins present in fraction III [2]. The proteins remaining in the supernatant were then precipitated with ethanol. The product thus prepared contains prealbumin, 20–25% IgM and 15–20% IgA. Combining this method with Aerosil and ion exchange resulted in a preparation that contains 4 mg IgM/ml or five times the concentration of IgM in normal plasma [3, 4]. Due to its pentamer structure, IgM is particularly suited to agglutinate bacteria. In a mouse model it was demonstrated that the IgM-containing preparation protects mice against *Salmonella* infection more effectively than a conventional intravenous IgG preparation [3]. In a recent clinical study, such a preparation containing 10–15% IgM was shown to be effective in the therapy of gram-negative sepsis [5]. Mortality in IgM-treated patients was 4 versus 32% in the control group. Thus, it is reasonable to believe that preparations of higher purity than the 10–15% IgM concentrate commercially available in Europe would be desirable from the standpoint of more rapid infusion and improved efficacy thereby increasing its therapeutic potential. In this paper, we will present a method of obtaining such a preparation. Specifically, we will discuss the classical euglobulin precipitation method where precipitation is controlled by pH and ionic strength. All procedures in the present study were designed to be compatible with production-scale sanitary application where continuous centrifugation is still a widely accepted technique in protein purification. We will also discuss process control with regard to inactivation on lipid-membrane-coated viruses and product complement activity by its ability to generate C4a. Classical pathway activation usually requires an involvement of immunoglobulins, immune complexes or immune aggregates. C4a generation signals particularly of the immune system in such a capacity as mediating inflammatory reaction [6]. And in addition, comparison of the physical and chemical properties of C4a with those of C3a and C5a establishes a high degree of structural similarity [7]. Therefore, an assay to measure C4a generating ability was developed to determine an acceptable level of anticomplement activity on IgM solution intended for infusion

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into an animal [8]. A potential vasodilator, prekallikrein activator (PKA), is also evaluated in the product. The proposed process has been scaled up for pilot plant operations with reproducible product characteristics

Materials and Methods

Purification Method

Figure 1 shows a flow diagram of the purification process. Fraction III paste used in this study was obtained frozen from a plasma fractionation plant (Clayton, N.C.). The paste (30 kg) was allowed to thaw overnight at 4 °C, after which it was solubilized in 12 vol of 0.05 *M* Na acetate buffer, pH 3.75, for 4 h at 25 °C. The solution was then stirred with 1.5% (v/v) octanoic acid, pH 4.65, for 4 h, cooled to 5 °C and settled overnight. Precipitate was removed by centrifugation in two tubular bowl centrifuges (Sharples AS16). The supernatant was clarified further by passage through 0.1 µm nominal filter cartridge. The clear solution was concentrated 10-fold and diafiltered against at least 5 vol water for injection. The operation was carried out in a 120-ft² Romicon ultrafiltration system with a nominal molecular weight cut-off value of 100,000. Completeness of diafiltration was checked by sampling the retentate and measuring the conductivity using an Orion conductivity-meter. Typically, the undiafiltered sample was 2.6 mmho/cm and the diafiltered sample was 0.05 mmho/cm. A sample was taken at this point for optimization studies. The salt-poor solution, typically at an A_{280} of 40–60, was then treated with a mixture of 0.3% tri-(n-butyl) phosphate (TNBP) and 1% Tween 80, pH 4.6–4.8, for at least 6 h at 25 °C for viral inactivation. The TNBP/Tween-80-treated solution was diluted with water for injection to a conductivity of less than 0.1 mmho/cm. Addition of water was needed because of the slight increase of ionic strength following pH adjustment to 4.6–4.8. Residual reactants from the viral inactivation step were removed and euglobulins were recovered by precipitation twice at pH 6.7 using 0.5 *M* NaOH, added at a rate of 20–30 mV/min. The paste recovered in a tubular bowl centrifuge (Sharples AS16), was prepared as a 5% protein solution in 10% maltose, pH 4.25 and held at 5 °C. Several batches could then be combined and heated at 50 °C for 1 h. Each batch was then diafiltered against at least 5 vol of 0.0025 *M* Na acetate, pH 4.25, in a Romicon ultrafiltration system with a nominal cut-off value of 500,000. The solution was formulated in 10% maltose, sterile filtered and filled. To provide an accelerated sterility check, the final containers were held at 25 °C for 21 days prior to storage at 2–10 °C.

C4a Generating Activity

The radioimmunoassay kit for human complement C4a des Arg (Amersham Inc., Arlington Heights, Ill., USA) was used. A pooled preparation of serum from at least 3 donors served as the complement source for activation. Modified from the procedure established by Wagner and Hugli [6], C4a generated was determined by measuring the ability of the sample to compete with a fixed amount of ^{125}I-labeled C4a des Arg tracer for a limiting quantity of rabbit anti C4a des Arg. All samples were tested at a constant concentration of 1.46 mg IgM/ml serum. The C4a generating activity was expressed in µg/ml of serum. Since there is a dilution of potential activated compounds in preparing the sample for this assay, a control sample, commercial intravenous γ-globulin solution, was prepared similarly.

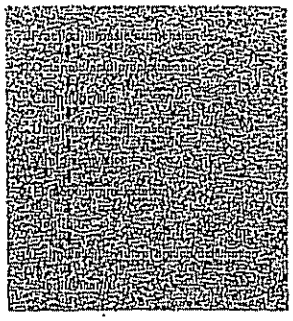

Fig. 1. IgM concentrate purification flow sheet.

Protein Concentration

In process protein concentration was determined by absorbance measurement at 280 nm and an extinction coefficient of 13.8. Total protein was calculated from the sample volume multiplied by protein concentration.

Quantitation of IgA, IgG and IgM

Quantitation of each immunoglobulin was determined by immunoprecipitation, with detection by infrared nephelometry. An automated Behring nephelometer (Behring Diagnostics, Somerville, N.J., USA) measured the sample turbidity due to light-scattering immune complexes between the antigen and a specific antiserum. Detailed description of the method is available in the manufacturer's instruction manual.

Quantitation of Tween 80

Sample was first deproteinized by 95% ethanol. Tween 80 remains in the supernate. Ethanol was evaporated at 50 °C and Tween 80 was redissolved in distilled water. The polyethoxylated compounds in Tween 80 formed a blue-colored complex with ammonium cobalthiocyanate reagent, which was soluble in dichloromethane. Optical density of the blue dichloromethane solution was measured at 620 nm. This optical density value was proportional to the concentration of Tween 80.

Viral Inactivation Studies

Vesicular stomatitis virus (VSV), a lipid-enveloped virus was chosen for this study. Titers were measured and expressed in terms of tissue culture infectious dose as a 50% endpoint per ml ($TCID_{50}$/ml) [9].

Quantitation of PKA

PKA activity was measured by a two-stage assay. It is based on the initial conversion by PKA of a partially purified prekallikrein substrate to kallikrein. This proteolytic enzyme is then assayed by its esterase action on the synthetic substrate, α-N-benzoyl L-arginine ethyl ester. The rate of α-N-benzoyl L-arginine ethyl ester hydrolysis is monitored by the change in optical absorbance of the mixture at 253 nm. The activity is expressed as a percentage of a Bureau of Biologics reference PKA preparation.

Results and Discussion

Effects of pH in Euglobulin Precipitation

Proteins become positively or negatively charged on either side of the isoelectric point, and these forms are more soluble than the electrically neutral molecule. The isoelectric point of IgM concentrate has been determined by isoelectric focusing to cluster around neutrality [unpubl. data]. Accordingly, IgM would precipitate from a solution at a pH range close to 7. In the experiment shown in figure 2, the pH of a diafiltered octanoic acid supernate (conductivity = 0.05 mmho/cm) at pH 4.6 was adjusted by 0.5 *M* NaOH to between 6.4 and 8.5. The precipitate was recovered and solubilized in an equal volume of water. Based on the A_{280} measurement, total protein was plotted against the pH at which precipitation occurred. The shape of the curve determined from 3 experiments reflects isoelectric behavior of the proteins with insolubility occurring between 6.4 and 7.5. From these results, it followed that the best recovery of the euglobulin is obtained at this pH range.

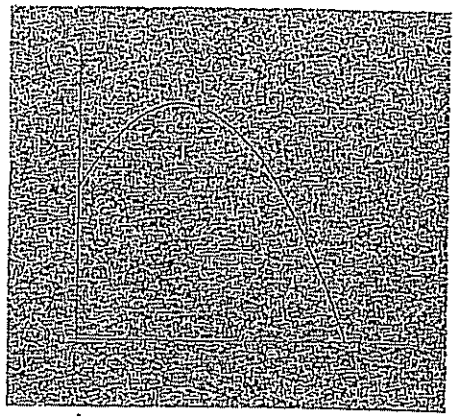

Fig. 2. IgM precipitation as a function of pH.

Effects of Ionic Strength in Euglobulin Precipitation

The diafiltered octanoic acid supernate was precipitated at pH 7.1 in solutions of four different ionic strengths, as designated by the molar concentrations of NaCl in figure 3. Each solution was centrifuged to remove the precipitate. As measured by infrared nephelometry, the amount of IgM, IgG and IgA remaining in the supernate represented the solubility of each globulin. It is evident that reduced solubility is associated with lower NaCl concentration, i.e., lower ionic strength. This relationship conforms to the Debey-Huckel theory [10] where solubility of globulin increases upon the addition of salts. Our results suggest that solubilities of polymeric immunoglobulins, both IgM and IgA, are very similar. Additionally, ionic strength exerted a more pronounced effect on the solubility of IgG. Thus, preferential precipitation of IgG from IgA and IgM occurs at low NaCl concentration.

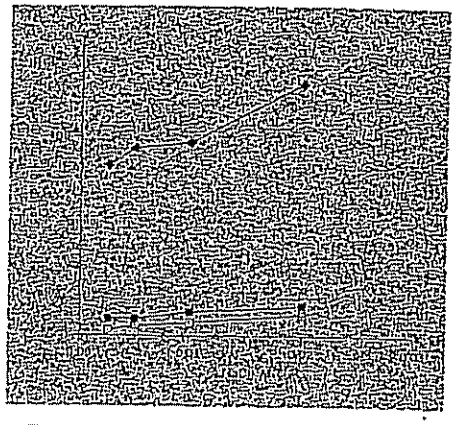

Fig. 3. Effects of ionic strength on the supernatant concentration after euglobulin precipitation. □ = IgM; ◆ = IgG; ■ = IgA.

Marker Virus Inactivation

Conditions for viral inactivation were similar to those developed by the New York Blood Center [9]. The inactivation of VSV added to an IgM solution containing 0.3% TNBP and 1.0% Tween 80 was studied over a pH range of 4.25–5.1. Results from a typical experiment are shown in figure 4. Relative to the untreated control titer of $10^{4.75}$ $TCID_{50}$/ml, minimal inactivation was seen at pH 5.1 for 3 h. This is contrary to previous observations with detergent/solvent treatment on AHF solutions at neutrality [9]. The difference could be due to much higher protein concentrations (A_{280} greater than or equal to 40) according to our purification scheme. It is in agreement with our in-house data that VSV infectivity is stabilized by proteins [unpubl. obs.]. Between pH 4.25 and 4.8, greater than 10^4 $TCID_{50}$/ml reduction in titer was achieved. At pH 4.25 and

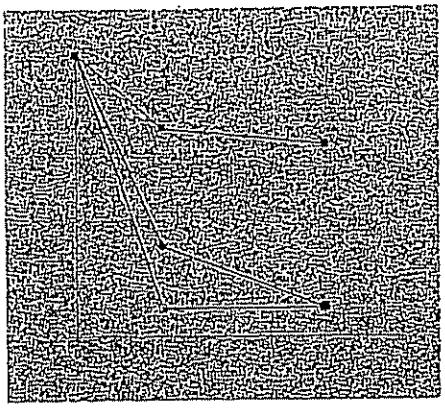

Fig. 4. Inactivation kinetics of VSV in IgM containing 0.3% TNBP and 1.0% Tween 80. □ = pH 4.25/4.4; ◆ = pH 4.8; ■ = pH 5.1.

Table 1. Preparation of 50% IgM concentrate

1	12.3	18.2	31.1	51	63
2	11.7	16.3	41.7	60	64
3	15.2	22.0	28.8	44	50
4	13.8	22.4	27.1	43	62
5	11.7	20.0	35.0	52	66
6	10.8	17.4	25.2	47	70
7	10.8	25.6	29.1	44	79
8	11.7	19.5	30.2	49	60
9	10.6	24.3	26.5	43	78
10	9.4	17.9	34.3	56	68
11	11.0	17.9	37.7	57	68
12	12.0	18.3	24.6	45	63
13	9.5	22.4	30.5	49	75
14	14.8	18.2	37.1	53	71
15	8.8	22.4	33.1	51	69
16	12.7	17.4	32.7	51	55

pH 4.4, inactivation was complete to the limit of detection after 1 h, the first time point assayed. While the use of pH 4.25 is preferred in terms of faster viral inactivation, it must be counterbalanced by the unacceptable increase in ionic strength when euglobulin precipitation is carried out by NaOH addition. Preliminary consideration thus suggested that a pH of 4.6–4.8 is a reasonable compromise, thereby achieving viral inactivation in 3 h and maintaining low ionic strength in the subsequent steps. It is noteworthy that our inactivation studies demonstrated that the acid condition alone was not the mechanism of inactivation since titers of the untreated controls at pH 4.6–4.8 were unaffected after 4 h.

Product Composition and Overall Yield

IgM concentrations in 16 preparations can be seen in table 1. IgM content in the concentrate was 50 ± 5% (n = 16). Each batch of fraction III paste was derived from a pool of over 3,000 plasma donors and, as a consequence, may exhibit little variation in the resulting immunoglobulin concentrations. Overall yield from the octanoic acid supernate to the final product averaged 66 ± 8% (n = 16).

Residual Reactants from Viral Inactivation

Considerable retention of TNBP/Tween 80 in the product was shown if precipitation was carried out only once after the viral inactivation step. TNBP, having a molecular weight of 266.3, is easily removed when the product is diafiltered. However, Tween 80 exists as detergent micelles and could bind to protein molecules by hydrophobic interactions [11]. Successful removal of traces of Tween 80 (< 25 ppm) is accomplished by reprecipitation after solubilizing the protein in a large volume of water. This volume was determined to be 40 vol equivalents of the protein precipitate (fig. 5). It can be observed from this figure that fewer volumes were not consistently effective in removing the residual Tween 80 from the protein solution.

Reduction of C4a Activity

When purified from human serum, globulin proteins (IgM, IgG, IgA) contain enzymatic as well as complement active proteins and other proteins as contaminants. One of the marker components in the complement-cascade chosen for this study is C4a-generating activity. Heat has been shown to reduce this activity in globulins. Reduction of this activity is a function of temperature and length of heating [12]. This is consistent with the observation that

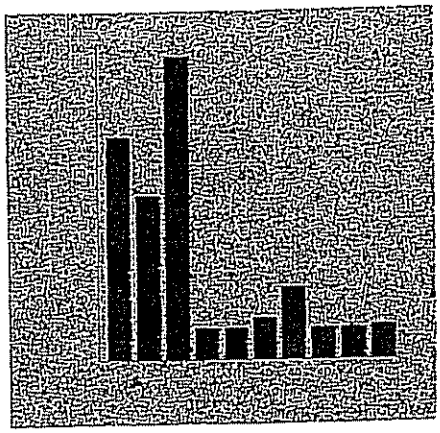

Fig. 5. Residual Tween 80 as a function of washes.

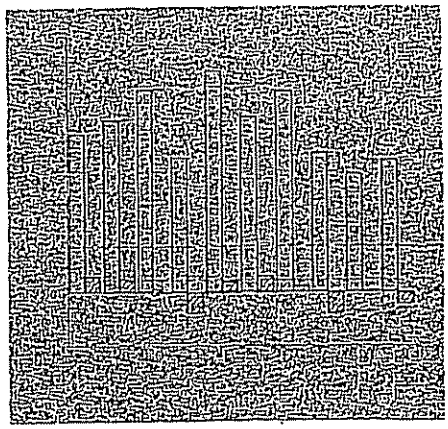

Fig. 6. C4a reduction across heating. □ = Preheated C4a activity of each lot in x-axis (pg/ml); ▨ = postheated C4a activity of each lot in x-axis (pg/ml); ---- = C4a activity in IGIV 0.1–0.93 pg/ml.

anticomplementary activity in intravenous human γ-globulin is reduced by incubating the solutions at pH 4 and 37°C for 8 h [13]. Upon heating at 50°C for 1 h, high C4a generating activity in unheated IgM preparations was greatly reduced to levels equal to or below those of a commercial intravenous γ-globulin preparation (fig. 6). Results obtained from HPLC and efficacy studies in mice indicated that the immunoglobulin was protected from denaturation due to heating [12].

PKA Activity in IgM Concentration

The most desirable IgM solution would be one that contains no potentially vasoactive agents. Lefer [14] has reviewed many cardioinhibitory factors, most of which are small peptides with molecular weights less than 10,000. If such factors are present, it is likely that the two diafiltration steps would aid in the removal of inhibitory effects. One identifiable vasodilator is pKA [15] which could trigger the generation of bradykinin. With a goal of below 20% of a bureau of Biologics reference PKA preparation, the study reported here demonstrates that fraction III can be processed to IgM concentrate which has a low amount of PKA (table 2).

Table 2. PKA activity in IgM concentrate

Lot No.	PKA
A	<1
B	8
C	1
D	<1
E	<1
PR 3167	<1
PR 3192	<1

Conclusion

We have reported a process aimed at the manufacture of an IgM concentrate from Cohn fraction III paste. Efficiency of euglobulin precipitation was largely determined by pH and ionic strength. Solvent-detergent treatment, the method of choice for viral inactivation in many plasma derivatives, was successfully applied to this product with a marker virus, VSV. IgM in the concentrate represents 50% of the immunoglobulins present. Clearly, process

reproducibility was demonstrated by meeting all criteria with regard to residual reactants, IgM concentration, PKA activity and C4a generating activity. While this kind of data is useful in guiding the development of an industrial process, it should be noted that significant issues associated with preclinical studies such as development of a suitable animal model must still be resolved. The capacity to produce large quantities of purified IgM should facilitate further biochemical studies of structure and function of this immunoglobulin.

Acknowledgment

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References

1 Cohn EJ, Strong LE, Hughes WL, Mulford DJ, Ashworth J, Melin M, Taylor HL: Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the proteins and lipoprotein components of biological tissues and fluids. J Am Chem Soc 1946;68:459–475.

2 Steinbrech M, Andrae R, Pejaudier L: Preparation of an IgM and IgA enriched fraction for clinical use. Prep Biochem 1973;34:363–373.

3 Stephan W: Intravenous IgM by means of treatment of Cohn fraction III with b-propiolactone; in Curling J (ed): Separation of Plasma Proteins. Uppsala, Pharmacia, 1982, pp 153–156.

4 Stephan W: Concentrated immunoglobulin solution suited for intravenous administration. United States Patent No. 4,318,902. March 9, 1982.

5 Schedel I, Dreikhausen U, Nentwig B, Hockenschieder M, Rauthmann D, Balikdioglu S, Coldewey R, Deicher H: Treatment of gram-negative septic shock with an immunoglobulin preparation – A prospective, randomized clinical trial. Crit Care Med 1991;19:1104–1113.

6 Wagner JL, Hugli TE: Radioimmunoassay for anaphylatoxins: A sensitive method for determining complement activation products in biological fluids. Anal Biochem 1984;136:75–88.

7 Gerlach JP, Hugli T, Muller-Eberhard HJ: Characterization of human C4a anaphylatoxin. J Biol Chem 1981;256:2707–2711.

8 Tary GC, Cheung N: Anticomplement activity assay. United States Patent, pending.

9 Horowitz B, Wiebe ME, Lippin A, Stryker MH: Inactivation of viruses in labile blood derivatives. I. Disruption of lipid-enveloped viruses by tri(n-butyl)phosphate detergent combinations. Transfusion 1985;25:516–522.

10 Green AA, Hughes W: Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents. Methods Enzymol 1955;10:67–90.

11 Green FA: Interactions of a nonionic detergent with soluble proteins. J Colloid Interface Sci 1971;35:481–485.

12 Tary GC, Jemmak G: Heat treated IgM preparation. United States Patent, pending.

13 Wickerhauser M, Hao YL: Large-scale preparation of immunoglobulins. Vox Sang 1972;23:119–125.

14 Lefer AM: Properties of cardioinhibitory factors produced in shock. Fed proc 1978;37:2734–2740.

15 Alving BM, Hojima Y, Pisano JJ, Mercan DL, Buckingham RS Jr, Mercan MM, Finlayson JS: Hypotension associated with prekallikrein activator (Hageman-factor fragments) in plasma protein fraction. N Engl J Med 1978;299:66–70.

EXHIBIT 15

Blood Coagulation and Fibrinolysis, 5 (3), 537–544

Review Paper

Virus validation of plasma-derived products produced by Pharmacia, with particular reference to immunoglobulins

B. Eriksson, L. Westman and M. Jernberg

Important factors to assure the safety of plasma-derived products manufactured on an industrial scale are initial screening of the source material and validation of the manufacturing process in accordance with issued EEC guidelines and US 'Points to Consider'. Pharmacia's manufacturing process for immunoglobulins contains a specific virucidal step, in which lipid-enveloped viruses are effectively inactivated with a solvent/detergent (SD) combination consisting of 0.3% tri(*n*-butyl)phosphate and 1% Tween 80. Results from virus validation studies of scaled-down versions of Pharmacia's manufacturing process for immunoglobulins demonstrated extensive removal of relevant and model viruses. More than 5.0 logs of human immunodeficiency virus type 1 (HIV-1) were inactivated in the SD step and, in total, more than 31 logs of HIV-1 were eliminated in the steps studied. Comparison between SD treatment and heating at 60°C of lipid-enveloped viruses in different protein solutions demonstrated that SD treatment is the superior procedure. Polio virus is a model often used in virus validation studies to predict effects on non-enveloped viruses. Because polio virus is more sensitive to heat than are hepatitis A virus (HAV) and human parvovirus B19, thermal inactivation studies with polio virus may result in an overestimation of the effects on HAV and B19.

Key words: Plasma, plasma fractionation, ethanol precipitation, immunoglobulins, virus safety, virus validation, solvent/detergent, pasteurization.

Introduction

Plasma-derived products are important biological therapies that both protect patients from acute life-threatening events and form a basis for efficient prophylactic treatment. As biological materials, plasma products are associated with an inherent risk of transmitting infectious agents that may be present in the source material. Due to insufficient sensitivity and the limitations of present diagnostic methods used in initial screening, donated plasma units cannot be assumed to be completely free of viruses. Although the majority of contaminated units can be detected and discarded, the source material obtained from collection centres and intended for fractionation may still be contaminated with undetected and infectious viruses. Therefore, methods for removal and inactivation of viruses play an important role in every purification process aimed to improve further the viral safety of plasma products.

Pharmacia manufactures several plasma products. Among coagulation factor concentrates, factor VIII (Octonativ-M) and factor IX (Nanotiv) are used in the treatment of haemophilia A and B. Other products of importance are antithrombin III (ATenativ), albumin and several forms of immunoglobulins.

In order to provide products with a high degree of viral safety, the manufacturing process contains at least one specific virus-inactivating step. In the case of Pharmacia's coagulation factor concentrates and all immunoglobulin products, specific virus inactivation is achieved by treatment with a combination of chemicals, namely a solvent, tri(*n*-butyl)phosphate (TNBP), and a detergent, Triton X-100 or Tween 80. The observation that this combination effectively inactivated viruses was originally made at the New York Blood Center and this procedure is today the most effective treatment available to inactivate lipid-containing

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enveloped viruses, such as the human immunodeficiency viruses types 1 and 2 (HIV-1, HIV-2), and hepatitis B and C viruses (HBV and HCV). The virus-inactivating methods used for albumin and ATenativ rely on the traditional pasteurization process, that is heating solutions of final product or intermediate material at 60°C for 10 h

Viruses in blood and plasma

Viruses that may be transmitted by blood and plasma products have been reviewed previously. Here, comments will be made on some of the viruses known to be of importance in the plasma fractionation industry.

Human retroviruses are represented by the devastating HIV-1 and HIV-2. Testing every donated unit of blood and plasma for the absence of antibodies to HIV-1 and -2 is mandatory. Mandatory testing for antibodies against two other human retroviruses, human T-lymphotropic viruses types I and II (HTLV-I and HTLV-II), has also been discussed in several countries and implemented by some. For HTLV-I and -II, transmission has been associated with transfusion of cellular blood products but not with cell-free plasma. This observation is strikingly different to the situation with HIV-1 and -2. On the basis of these observations, a majority of national agencies considers that there is presently no reason to initiate screening of plasma for evidence of HTLV-I infection. Further studies are warranted to confirm whether HTLV particles may exist outside the cell and whether these particles, if found, are really non-infectious.

A second large group of pathogenic viruses consists of the human hepatitis viruses. These belong to fundamentally different virus families and therefore have very different characteristics. Every donated unit of blood and plasma is screened for the presence of hepatitis B surface antigens (HBsAg) and HCV antibodies. The extent to which hepatitis A virus (HAV) is a significant problem associated with donated plasma is still under discussion. In contrast to HBV and HCV, HAV is a small, non-enveloped virus and, as such, is also significantly more resistant to inactivation. During the past 3–4 years, a number of haemophiliacs in Italy, Germany, Belgium and Ireland have become infected by HAV during treatment with several batches of contaminated factor VIII concentrates from different production plants but originating from one manufacturer's process. It should be kept in mind that HAV transmission has not been reported for any other factor VIII concentrate or for any other plasma product on the market. A thorough investigation was attempted to find the reason for this contamination, but no clear-cut answer could be found. A combination of factors seemed to have been responsible for this spread of HAV, including sources not related to contaminated plasma. These incidences did, however, highlight a potentially insufficient viral safety margin for that particular manufacturing process with respect to HAV and issued warning signals for similar processes and products.

Human parvovirus B19 is another example of a so-called naked virus that may be transmitted by blood and plasma products. As with HAV, the vast majority of B19 infections are transmitted by routes other than through blood or plasma, and B19 infections are normally acquired early in life. The occurrence of a high-titre viraemic phase in infected individuals, although of short duration, means that there is a risk of transmitting this infection by donated blood and plasma.

Safety of manufacturing processes

The manufacture of sufficiently safe plasma-derived products that meet today's state-of-the-art demands on viral safety relies on at least three levels of intervention. First, the source material is selected by following specific donor programmes and by screening donated plasma to eliminate virus-positive units. Secondly, the manufacturing process plays an important role in eliminating, separating and inactivating viruses that may not have been detected by initial screening. Finally, attempts are also made to determine whether the finished product or its intermediates are completely free of viruses. No single approach alone will guarantee that the finished product will be safe. The manufacturing process and its design are the most important individual factors in the three approaches mentioned above. The reason for this is simply that even the best available screening tests for antibodies fail to detect infected units due to the window period. The current risks of a false-negative result in the USA are about 1 in 225 000 for HIV-1 and about 1 in 6 000 for HCV when second generation tests are used. Because the manufacturing process contains several chromatographic separation steps and one or several very potent and specific virus-inactivating steps, the elimination of significant amounts of viruses can be achieved, which may guarantee a higher degree of safety than is possible by initial screening alone. According to the Committee for Proprietary Medicinal Products (CPMP) Note for Guidance, Validation of Virus Removal and Inactivation Procedures,[1] the documented overall virus reduction achieved for relevant viruses in validation studies of a manufacturing process should be substantially greater than the maximum possible virus titre that could potentially occur in the source material.

Achieving levels that eliminate an additional 3–5 logs of virus above the theoretical virus burden in source material is considered an acceptable safety margin, but this needs to be considered on a case-by-case basis.

Validation of virus inactivation

A major issue in virus-validation studies deals with the choice of viruses. The choice and number of viruses used in validation studies is dependent on the quality and origin of the source material. Two categories of viruses are discussed in this context. So-called relevant viruses are viruses that can be detected in, or are likely contaminants of, the source material. HIV is one example of a relevant virus for which it is mandatory to test in all validation studies of processes that use human plasma. The second category comprises different model viruses that are assayed when no or insufficient *in vitro* infectivity assays exist for relevant viruses. HCV, for example, has not yet been cultivated in and isolated from cell culture. Therefore, a closely related virus has to be used for which good detection assays are available in order to estimate the potential effects of an inactivation or separation process on HCV. The models often used are bovine viral diarrhoea virus, tick-borne encephalitis virus, yellow fever virus and Sindbis virus. Also of importance is the inclusion of a non-enveloped virus in virus validation to predict the effect on such viruses. Recently, cell-culture models of HAV have become available. By using either the HAV or an animal parvovirus model, more reliable results may be obtained for this category of viruses than with polio virus. Polio virus is more susceptible to heat inactivation than either HAV[2,3] or parvoviruses (unpublished observations). If results from polio virus studies are used to predict effects on HAV or human parvovirus B19, an overestimation of the inactivation effect may be made. Additional information and comments on viruses that are normally used in validation studies have been summarized in several publications.[4–6]

Guidance to the performance of virus validation studies is found in the recommendations from National Agencies.[1] Additional technical and theoretical considerations on the execution of virus-validation studies and the associated difficulties in assaying samples of low viral content have been discussed in several papers.[7,8] Briefly, virus of high titre is used to spike the starting material of a particular process step. The process is simulated in a laboratory scaled-down version. Determinations of the amounts of virus in the starting material and in different fractions of the material obtained after the process step are carried out in infectivity assays. The so-called virus-reduction factor is defined as the $\log_{10}$ of the ratio of the virus load in the pre-purification material divided by the virus load in the post-purification material. If individual steps of a process inactivate and remove viruses by independent mechanisms, the reduction factors for these steps can be added to obtain an overall reduction factor for the complete process.

Virus inactivation in the manufacture of immunoglobulins

Figure 1 shows a flow-chart of the purification process for immunoglobulins manufactured at Pharmacia. This process can be divided into three major stages. Initially, plasma is subjected to a series of ethanol-precipitation steps, referred to as a modified Cohn-Deutsch procedure, which also includes separation over a DEAE–Sephadex column. Ethanol contributes to the elimination of viruses, both by partitioning and by a direct virucidal effect. In the second stage, the DEAE–Sephadex-treated fraction II material is subjected to a specific virus-inactivating treatment with 0.3% TNBP and 1% Tween 80 for 6 h. This combination is extremely effective against all known lipid-enveloped viruses. The solvent and detergent are removed from the immunoglobulins by oil extraction and CM–Sepharose chromatography. The third stage of importance for the inactivation of viruses is the low pH treatment prior to the last chromatography step.

Several of the individual steps have been evaluated for their inhibitory and/or separation effects using HIV-1, Sindbis virus and vesicular stomatitis virus (VSV). Table 1 summarizes the individual and overall reduction factors obtained for these viruses in recent studies. The most important virus-inactivating effect is produced by TNBP and Tween 80. Total inactivation of all of the three studied viruses was achieved within a fraction of the total treatment period. As an example from one of the inactivation studies, the time-dependent kinetic pattern of VSV is shown in Figure 2. A complete inactivation of ≥ 4.8 logs of virus was achieved within 1 h of exposure in this experiment. For all viruses studied, better reduction values are difficult to obtain because the titre of the original virus stock is not only diluted ten-fold in the protein solution but also further diluted 100- to 1000-fold to lower the cell-toxic effects of the chemicals and to arrest the virus-inhibitory effects of TNBP and Tween in order to measure remaining virus infectivity. The elimination of ≥ 4.8 logs of VSV corresponds to an original virus stock titre of $10^{7.8}$ $TCID_{50}$/ml. $TCID_{50}$ is one way of expressing virus infectivity units and stands for the tissue culture infectious dose required to cause a virus infection in 50% of inoculated cultures.

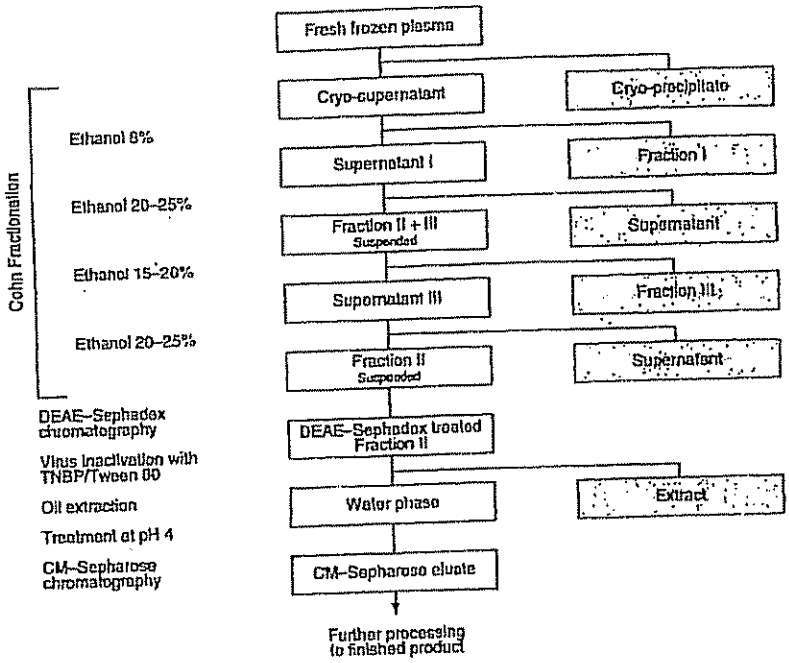

Figure 1. Schematic representation of the individual steps in the manufacturing process of immunoglobulins at Pharmacia

The inclusion of a specific virus-inactivating method in the manufacturing process of immunoglobulins has been discussed over several years. Recently, the importance of a specific virus-inactivating method was further strengthened by observations made by Yei and colleagues.[1] They reported that when immunoglobulins were prepared from a pool of HCV-positive plasma, in which infectivity was quantified by polymerase chain reaction technology, most of the HCV RNA partitioned into cryo-precipitate, fraction I and fraction III, but trace amounts could still be detected in fraction II material, which is further processed to immunoglobulins. Although HCV RNA is not synonymous with infectious virus, the existence of viral RNA in fraction II clearly demonstrates that the ethanol-precipitation steps cannot effectively clear HCV from immunoglobulin products and may explain some of the incidences of HCV transmission that have occurred. In contrast to intravenous immunoglobulins, transmission of HCV has never been reported for intramuscular immunoglobulins. The most attractive explanation for this discrepancy is the different route of administration and the fact that smaller doses are given. However, there may be a potential risk for virus transmission if the product enters the blood system. An evaluation of the risks associated with subcutaneous administration has not yet been made. In the light of still sporadic reports of HCV transmission by intravenous immunoglobulins, a specific virus-inactivating step should be considered in future for all immunoglobulin products.

The precipitation of fraction II + III material using 25% ethanol at sub-zero temperatures resulted in the elimination of more than 10 logs of infectious HIV-1 (Table 1). Strikingly different results were obtained for VSV and Sindbis virus which were neither significantly

Table 1. Summary of reduction factors obtained from validation studies of the immunoglobulin manufacturing process at Pharmacia

Process step	Reduction of virus infectivity (log_{10})		
	HIV-1	VSV	Sindbis
Cryo-separation	> 1.0	n.d.	n.d.
Cohn fractionation			
Ethanol 8%	> 2.1	n.d.	n.d.
Ethanol 25% of 'Sup I' (Fractions II + III)	> 10.0	0.5	0.9
Ethanol 25% of 'Sup III' (Fraction II)	3–4	n.d.	n.d.
Solvent/detergent (TNBP/Tween 80)	> 5.0	> 4.0	> 5.2
Other steps			
Treatment at pH 4.0	> 10.0	> 4.0	> 5.0
Accumulated reduction factors	> 31.1	> 8.0	> 10.2

n.d., not determined.

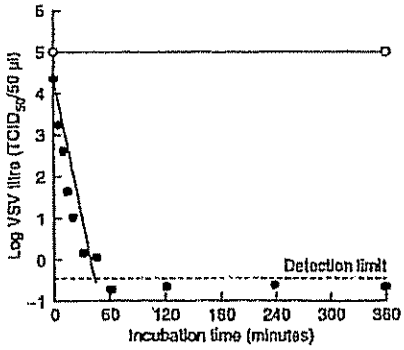

Figure 2. Time-dependent inactivation of vesicular stomatitis virus (VSV) by 0.3% TNBP/1% Tween 80 (●) at room temperature in an immunoglobulin solution containing 42 mg/ml protein. The control was VSV in the same solution without solvent/detergent (○).

inactivated by ethanol nor separated by precipitation. The results obtained with control procedures demonstrated that HIV-1 was largely inactivated by ethanol. The effect of ethanol on HIV-1 and other viruses has been reviewed by Morgenthaler.[12] Observations reported by Hénin *et al.*[13] demonstrated that ethanol causes substantial but slow inactivation of HIV-1 at −5°C. However, previous observations by Piszkiewicz *et al.*[14] demonstrated that HIV-1 infectivity fell rapidly by more than 3.5 logs in 5 min upon exposure to 20% ethanol at −5°C. Also, Dorner *et al.*[15] have reported a reduction factor for HIV-1 of more than 7 logs during this step in their purification process. Significant inactivation of HIV-1 by 20% ethanol has been observed when studies were performed at room temperature.[16] In the authors' studies, all samples were desalted before infectivity assays were performed. The reason for this was to prevent the indicator cells being affected by a significant amount of salt and also to maintain a uniform composition of all our samples when neat dilutions were assayed. The desalting procedure could not be performed at subzero temperatures because this would cause samples to freeze. The short duration during which HIV-1 in ethanol solution was kept at a slightly higher temperature may have affected virus infectivity to some extent before the exchange of 25% ethanol for medium was completed. A contributing factor that allowed a good elimination of HIV-1 was that the study was performed on a 500 ml scale; this allowed the addition of $10^{10.5}$ $TCID_{50}$ units of infectious HIV-1, which was completely inactivated. Additional factors that may have contributed to the elimination of HIV-1 were the use of 25% ethanol, a concentration slightly higher than those used in the previous studies cited above, and the fact that the protein solution may have contained other components that could have affected the HIV-1 infectivity.

It was shown that between 3 and 4 logs of HIV-1 infectivity were inactivated during precipitation of fraction II. Again, complete inactivation of HIV-1 was observed. The discrepancy between observations made in this and the above studies[12–16] clearly illustrates the difficulty in comparing results of validation studies. In this case, the study was performed on a smaller scale a few years earlier, using several clinical isolates of HIV-1, which were not concentrated to high titres. Consequently, this left no possibility of determining greater reduction values.

It is well known that HIV-1 is rapidly inactivated at extreme pH values. Observations have been reported that reduction by 3 logs was achieved after 10 min when the pH was lowered from 7.0 to 5.7.[16] A complete loss of HIV-1 infectivity in IgG solution within 2 h of treatment at pH 4.0 has also been observed by Kempf *et al.*[17] At least 90 min were required in the authors' studies with DEAE-treated fraction II material kept at pH 4.0, to accomplish a complete inactivation of

HIV-1 with a starting titre of $10^{9.5}$ $TCID_{50}$/mL. With an assay detection limit of $10^{0.5}$, the total reduction of HIV-1 infectivity was at least 10 logs. Also, VSV and Sindbis virus were significantly inactivated in similar studies during a 3-h exposure, resulting in reduction values of about 4 logs and 5 logs, respectively. The inactivation pattern was initially very rapid but became more complex with time, resulting in a significant plateau. Similar observations have also been reported for VSV and Semliki Forest virus (SFV) by Hämäläinen *et al.*,[18] although the present observations indicated a more efficient inactivation of VSV with time.

With regard to Pharmacia's immunoglobulins, it has been demonstrated that the overall reduction factor for HIV-1 infectivity is more than 31 logs in the studied steps. Corresponding values for VSV and Sindbis virus were more than 8.0 logs and more than 10.2 logs, respectively.

Validation of the process with respect to non-enveloped viruses is under way. The inherent risk of HAV transmission by immunoglobulins must be considered minimal. This product, like albumin, has been widely used for decades and has never been associated with transmission of this disease. Immunoglobulins have, in contrast, been used as a prophylactic treatment to passively immunize and protect against HAV infection. However, HAV may be present in the plasma pool. As with human parvovirus B19, it is believed that HAV should be effectively neutralized if antibodies are present. Minor separation will occur during ethanol precipitation steps, with the exception of precipitation of fraction III. At this step, a significant removal of viruses may be accomplished. Some separation may also be achieved during the chromatography steps.

Solvent/detergent treatment and pasteurization

The two most documented and reliable virus-inactivating methods available today depend on solvent/detergent (SD) treatment and pasteurization. SD treatment inactivates all lipid-enveloped viruses and leaves the desired proteins unaffected, whereas heating in solution requires the addition of stabilizers to protect the protein under purification. The background to and the development of the SD process as a selective and effective alternative to heating has been described previously by Horowitz *et al.*[17] During the initial studies at the New York Blood Center, it was shown that the SD treatment did not affect non-enveloped viruses, such as encephalomyocarditis virus.[19] If the presence of non-enveloped viruses, such as HAV and human parvovirus B19, is to be considered a future problem associated with plasma, new and more selective inactivation methods against such viruses are urgently needed. Pasteurization is effective against non-enveloped viruses to some extent, but can be used only under circumstances where proteins under purification are sufficiently protected against heat denaturation. A stabilized environment will unfortunately also protect viruses from being efficiently inactivated. Furthermore, there is a clear risk of overestimating the inactivating effect of heating on HAV and human parvovirus B19 if polio or any other virus is used which is more heat labile than are the former viruses. Unpublished observations on the inactivation of porcine parvovirus indicate that the effect of heating in solution at 60°C for 10 h inactivates only about 2 logs of virus, which is an elimination effect corresponding to that accomplished by separation using one regular chromatography step.

Although the two major virus-inactivating methods have been established for several years, there are very few data from studies attempting to compare the methods. Horowitz *et al.*[19] and Long *et al.*[21] compared the effect of 1% TNBP and 1% Triton X-100 with the effect of heating at 60°C in studies using duck hepatitis B virus (DHBV) in factor VIII concentrates stabilized with 50% sucrose and 2.2 mol glycine/l. The results demonstrated that the SD combination was superior to heating and inactivated at least 6.0 logs of DHBV in 90 min. Complete inactivation of at least 7.3 logs was achieved in less than 4 h. In contrast, heating inactivated about 5 logs over the entire 10-h treatment period.

Immunoglobulin solutions require addition of significant amounts of stabilizers to make possible a comparison between SD treatment and heat inactivation. The authors therefore used an intermediate protein solution from the antithrombin III manufacturing process with a composition that can be pasteurized leaving the antithrombin III molecule intact, and determined which of the two methods would be more effective against HIV-1 and Sindbis virus after addition of virus to similar solutions. As shown in Figure 3, HIV-1 was totally and more or less instantaneously inactivated by 0.3% TNBP and 1% Triton X-100 treatment, whereas heating at 60°C required about 30 min to achieve a similar level of inactivation. Similarly, Sindbis virus was totally inactivated within minutes of exposure to the SD combination, whereas heating required about 2 h of exposure for complete elimination (Figure 3). The use of pasteurization in the manufacture of albumin and antithrombin III by probably every manufacturer has provided considerable and impressive clinical documentation that products of sufficient viral safety can be manufactured using this treatment. However, in the light of the present results, it can be concluded that

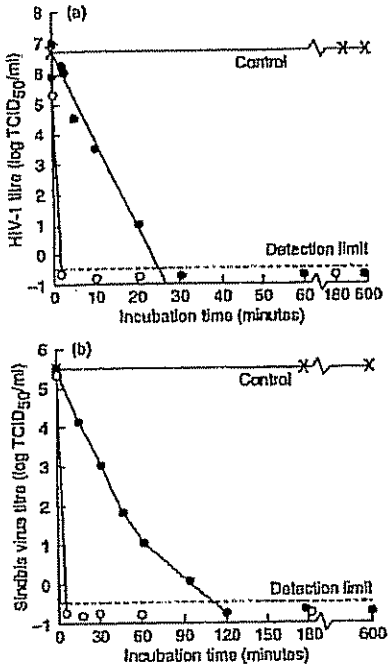

Figure 3. Time-dependent inactivation of (a) HIV-1 and (b) Sindbis virus in anti-thrombin III intermediate solution by heating (●) at 60°C or by treatment with 0.3% TNBP/1% Triton X-100 at room temperature (○). Controls of HIV-1 (X) and Sindbis virus (X) were kept at room temperature in solutions lacking solvent/detergent during the entire treatment period.

SD treatment using 0.3% TNBP and 1% Triton X-100 is not only a superior method but also one that gives the greatest margin of viral safety in the solutions studied.

Conclusions

Pharmacia's manufacturing process for different immunoglobulins contains a specific virus-inactivating step based on SD treatment. In comparison with traditional pasteurization, SD treatment is the superior method to inactivate lipid-enveloped viruses such as HIV, HBV and HCV. An equally effective method to inactivate non-enveloped viruses does not yet exist. Our experience with parvovirus indicates that inactivation by pasteurization is comparable with the effect of separation normally achieved using an ion-exchange chromatographic procedure.

References

1. Committee for Proprietary Medicinal Products: ad hoc Working Party on Biotechnology/Pharmacy and Working Party on Safety of Medicines. EEC regulatory document, note for guidance, validation of virus removal and inactivation procedures. *Biologicals* 1991; 19: 247–251.
2. Parry JV, Mortimer PP. The heat sensitivity of hepatitis A virus determined by a simple tissue culture method. *J Med Virol* 1984; 14: 277–283.
3. Siegl G, Weitz M, Kronauer G. Stability of hepatitis A virus. *Intervirology* 1984; 22: 218–226.
4. Vicari G. Validation of purification procedures for removing and/or inactivating viruses in biologicals: points to consider. In: Horaud F, Brown F, eds. *Virological aspects of the safety of biological products. Developments in biological standardization.* Basel: Karger, 1990; 75: 227–232.
5. Grun JB, White EM, Sito AF. Viral removal/inactivation by purification of biopharmaceuticals. *BioPharm* 1992; Nov–Dec: 22–30.
6. Suomela H. Inactivation of viruses in blood and plasma. *Transfusion Med Rev* 1992; VIII: 42–57.
7. Hilfenhaus J, Niedrig M, Nowak T. Theoretical and technical concerns in inactivation/elimination of viruses in plasma derivatives. In: Brown F, ed. *Virological safety aspects of plasma derivatives. Developments in biological standardization.* Basel: Karger, 1993; 81: 117–123.
8. Minor PD. Criteria for the choice of viruses in validation studies. In: Brown F, ed. *Virological safety aspects of plasma derivatives. Developments in biological standardization.* Basel: Karger, 1993; 81: 215–219.
9. Willkommen H, Löwer J. Theoretical considerations on viral inactivation or elimination. In: Brown F, ed. *Virological safety aspects of plasma derivatives. Developments in biological standardization.* Basel: Karger, 1993; 81: 109–116.
10. Darling AJ. Considerations in performing virus spiking experiments and process validation studies. In: Brown F, ed. *Virological safety aspects of plasma derivatives. Developments in biological standardization.* Basel: Karger, 1993; 81: 221–229.
11. Yei S, Yu MW, Tankersley DL. Partitioning of hepatitis C virus during Cohn–Oncley fractionation of plasma. *Transfusion* 1992; 32: 824–828.
12. Morgenthaler J-J. Effect of ethanol on viruses. In: Horaud F and Brown F, eds. *Virological safety aspects of plasma derivatives. Developments in biological standardization.* Basel: Karger, 1993; 75: 109–121.
13. Hénin Y, Maréchal V, Barré-Sinoussi F, *et al.* Inactivation and partition of human immunodeficiency virus during Kistler and Nitschmann fractionation of human blood plasma. *Vox Sang* 1988; 54: 78–83.
14. Piszkiewicz D, Kingdom H, Appelzweig R, *et al.* Inactivation of HTLV-III/LAV during plasma fractionation. *Lancet* 1985; ii: 1188–1189.
15. Dorner F, Barrett PN, Eibl J. At what stage should virus inactivation be carried out? In: Brown F, ed. *Virological*

safety aspects of plasma derivatives. Developments in biological standardization. Basel: Karger, 1993; 81: 137–143.
16. Spire B, Barré-Sinoussi F, Montagnier L, *et al.* Inactivation of lymphadenopathy associated virus by chemical disinfectants. *Lancet* 1984; ii: 899–901.
17. Kempf C, Jentsch P, Barré-Sinoussi F, *et al.* Inactivation of human immunodeficiency virus (HIV) by low pH and pepsin. *J Acquir Immune Defic Syndr* 1991; 4: 828–830.
18. Hämäläinen E, Suomela H, Ukkonen P. Virus inactivation during intravenous immunoglobulin production. *Vox Sang* 1992; 63: 6–11.
19. Horowitz B, Wiebe ME, Lippin A, Stryker MH. Inactivation of viruses in labile blood derivatives. I Disruption of lipid-enveloped viruses by tri(*n*-butyl) phosphate detergent combinations. *Transfusion* 1985; 25: 516–522.
20. Horowitz B, Prince AM, Horowitz MS, Watkleviex C. Viral safety of solvent-detergent treated blood products. In: Brown F, ed. *Virological safety aspects of plasma derivatives. Developments in biological standardization.* Basel: Karger, 1993; 81: 147–161.
21. Long Z, Sun C-S, White EM, Horowitz B, Sito AF. Hepatitis B viral clearance using duck virus model. In: Brown F, ed. *Virological safety aspects of plasma derivatives. Developments in biological standardization.* Basel: Karger, 1993; 81: 163–168.

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(54) Verfahren zur Herstellung von virusinaktivierten Immunglobulinlösungen.

(57) Die vorliegende Erfindung beschreibt ein Verfahren zur Herstellung von zur intravenösen Applikation geeigneten virusinaktivierten Immunglobulinlösungen, dadurch gekennzeichnet, daß das Immunglobulin mit nichtionischen Tensiden behandelt wird, die anschließend durch Festphasenextraktion an hydrophoben Materialien entfernt werden.

EP 0 525 502 A1

Gegenstand der Erfindung ist ein Verfahren zur Herstellung virusinaktivierter Immunglobulinlösungen, die zur intravenösen Injektion geeignet sind.

Immunglobuline sind humorale Glykoproteine, die bei der Elektrophorese der Plasma- bzw. Serumproteine in der sogenannten Gamma-Fraktion wandern und daher früher als Gamma-Globuline bezeichnet wurden.

Immunglobuline werden wegen ihres hohen Gehalts an Antikörpern zur Prophylaxe und Therapie von Infektionen verwendet.

Es ist bekannt, Immunglobuline sowohl für die intramuskuläre als auch subkutane Applikation herzustellen. Eine vielfach angewendete Herstellungsmethode ist die sogenannte Cohn-Oncley-Fraktionierung, auch 6/9-Methode genannt (Cohn et al., J. Am. Chem Soc. 68, 459 (1946); Oncley et al; J. Am. Chem. Soc. 71, 541 (1949).

Dieses Herstellungsverfahren hat jedoch den Nachteil, daß es zu einer hochviskosen Lösung, die nur intramuskulär oder subkutan applizierbar ist, mit hohen Antikörperkonzentrationen in relativ geringen Volumina führt. Das erhaltene Produkt ist zwar bei 4°C stabil, es kann jedoch Proteolyse durch Plasmaverunreinigungen auftreten. Weiterhin können IgA- und IgG-Dimere vorhanden sein, die bei der Applikation zu einer anaphylaktischen Reaktion der Patienten führen können (Ullmann, Enzyklopädie of Industrial Chemistry, 1989, A14, Seiten 93. 94)

Aus diesem Grunde sind intravenös applizierbare Immunglobuline entwickelt worden, die beim Patienten eine bessere Verträglichkeit zeigen. Diese werden aus der sogenannten Fraktion 3 oder auch Cohn-Fraktion 2 (Cohn et al. a. a. O.) bei pH4 unter Verwendung von Polyethylenglycol, einer anschließenden Ethanolfällung Ultra- oder Diafiltration und Ionenaustauscherchromatographie hergestellt. Das auf diese Art erhaltene Immunglobulin wird mit Mono- oder Disacchariden stabilisiert.

Ein solches verbessertes Verfahren wird in der EP 0 073 371 beschrieben.

Ausgehend von der Fraktion 3 (Cohn et al. a. a. O.) wird nach Lösen und Einstellung des pH-Wertes auf pH4 eine Ultrafiltration und eine Diafiltration vorgenommen. Anschließend wird das erhaltene Filtrat zu einem Proteingehalt von 5 Gew.-% konzentriert und der Alkoholgehalt auf 8 Gew.-% reduziert. Nachdem die so erhaltene Immunglobulinlösung auf einen Proteingehalt von 8% aufkonzentriert ist, erhält man eine klare wasserartige Lösung mit einer Ionenstärke von 0,01 und einem pH-Wert von 4,2. Die Tonizität der Lösung wird mit 10 Gew.-% Maltose bei einem Proteingehalt von 5 Gew.-% eingestellt. Anschließend wird sterilfiltriert und lyophilisiert. Das lyophilisierte Material wird vor der Injektion in geeigneten Medien gelöst.

Ein großer Nachteil der so hergestellten Immunglobuline zur intravenösen Anwendung ist, daß sie vor Gebrauch erst in geeigneten Medien gelöst werden müssen und nur in lyophilisierter Form lagerfähig sind.

Ein weiterer Nachteil liegt darin, daß bei Anwendung dieser Immunglobuline Viren auf den Patienten übertragen werden können, da während des Herstellungsverfahrens keine Virusinaktivierung stattfindet. So wurde nach intravenöser Immunglobulingabe über Hepatitis-Erkrankungen und HIV-Infektionen berichtet (Ullmanns Encycl of Ind. Chem., Vol. A14, 1989, pages 102, 103).

Das technische Problem der Erfindung war es daher, ein Verfahren zur Virusaktivierung von intravenös injizierbaren Immunglobulinen zu entwickeln, das zu einem Produkt führt, bei dessen Applikation keine Virusübertragungen auf den Patienten auftreten und welches so stabil ist, daß es selbst ohne Lyophilisierung direkt als Injektionslösung hergestellt und gelagert werden kann.

Das technische Problem der Erfindung wird durch ein Verfahren gelöst, das dadurch gekennzeichnet ist, daß das Immunglobulin mit nichtionischen Tensiden behandelt wird, die anschließend durch Festphasenextraktion an hydrophoben Materialien entfernt werden.

Als nichtionische Tenside werden insbesondere TNBP und/oder Triton X 100 verwendet. Der pH-Wert der Lösung beträgt vorzugsweise 5,0 bis 5,5.

In einer bevorzugten Ausführungsform wird nach der Behandlung mit nichtionischen Tensiden mit biologisch kompatiblen Pflanzenölen extrahiert und diese dann abgetrennt. Als Pflanzenöle werden bevorzugt Rizinusöl oder Sojaöle verwendet.

Die anschließende Festphasenextraktion erfolgt in bevorzugter Weise mit octadecylderivatisierten Stoffen, die auch für die Umkehrphasenchromatographie verwendet werden. In einer besonders bevorzugten Ausführungsform erfolgt die Festphasenextraktion durch eine Umkehrphasenchromatographie an Octadecyl-(C-18)-Harz.

Dem fertigen Produkt kann nach der Festphasenextraktion ein Disaccharid zur Stabilisierung zugegeben werden. Die fertige Lösung wird dann ein oder mehrmals sterilfiltriert.

Weiterhin kann die Immunglobulinlösung vor der Virusaktivierung und/oder nach der Sterilfiltration einer Ultra- und Diafiltration unterzogen werden.

Im folgenden wird das erfindungsgemäße Herstellungsverfahren im einzelnen beschrieben. Zunächst wird, wie aus dem Stand der Technik bekannt, die sogenannte Cohn-Fraktion II in Wasser gelöst, bis eine vollständige klare Lösung erhalten wird. Die Lösung wird dann auf einen pH-Wert von 4,0 bis 5,0, vorzugsweise 4,5, eingestellt und zur Entfernung von Verunreinigungen filtriert.

Anschließend wird mit der Lösung eine Ultrafiltration durchgeführt und die Lösung ankonzentriert.

Die Ausschlußgrenze der Ultrafiltration ist 30.000 Dalton. In diesem Schritt werden insbesondere Verunreinigungen mit niedrigen Molekulargewichten entfernt. Daran anschließend wird eine Diafiltration zur Entfernung von Ionen vorgenommen, woran sich die eigentliche Virusinaktivierung anschließt.

Hierzu wird die Lösung zunächst abgekühlt auf 4 bis 8 °C und der pH-Wert auf 5,0 bis 5,5 vorzugsweise 5,3, eingestellt. Es werden dann nichtionische Tenside vorzugsweise TNBP und/oder Triton X 100, zugegeben und diese Lösung dann mehrere Stunden gerührt. Im Anschluß daran kann in einer bevorzugten Ausführungsform eine Pflanzenölextraktion vorgenommen werden. Hierbei werden der Lösung 5 Gew.-% Pflanzenöl zugegeben, die Lösung anschließend auf Raumtemperatur gebracht und mit dem Pflanzenöl verrührt. Der anschließenden Phasentrennung wird eine Filtration angeschlossen.

Die Lösung wird dann auf eine C 18-Säule gegeben und chromatographiert. Nach der Chromatographie erfolgt eine pH-Werteinstellung auf pH 4. Zur Einstellung der Tonizität wird Maltose zugegeben. Nach der anschließenden Sterilfiltration wird eine Stabilitätsprüfung in der so erhaltenen Lösung vorgenommen, indem diese mindestens 22 Stunden bei 37°C aufbewahrt wird. Zeigt die Lösung Trübungen, ist sie nicht verwendbar. Zeigt die Lösung in dieser Zeit keine Trübung, wird der pH-Wert auf 5,0 bis 5,5, vorzugsweise 5,3, eingestellt, eine weitere Ultra- und Diafiltration vorgenommen und die so erhaltene Lösung nach Zugabe von Maltose auf einen Proteingehalt von 50 g/l eingestellt. Anschließend wird eine weitere Sterilfiltration vorgenommen und direkt in Infusionsflaschen abgefüllt.

Das so erhaltene Produkt kann direkt intravenös injiziert werden und ist frei von Viren.

Das folgende Ausführungsbeispiel soll das erfindungsgemäße Verfahren näher erläutern.

Ausführungsbeispiel

Die Cohn-Fraktion II wird mit der sechsfachen Menge Wasser gelöst und so lange gerührt, bis eine klare Lösung erhalten wird. Anschließend wird der pH-Wert mit 0.5 normaler HCl auf 4 5 eingestellt. Es schließt sich zunächst eine Ultrafiltration an, bei der die Lösung auf 90 g/l ankonzentriert wird. Als Membrantyp wird Novasette 30 K verwendet. Daran anschließend wird mit der fünffachen Menge Wasser verdünnt und bei 0.3 bis 0,5 bar eine Diafiltration vorgenommen. Nach dieser Diafiltration wird die diafiltrierte Lösung auf 70 g/l Proteingehalt eingestellt. Die Lösung wird auf 4 bis 8 °C abgekühlt und auf einen pH-Wert von 5,3 mittels 0.1 normaler Natronlauge eingestellt. Anschließend wird 0.3 Gew.-% TNBP und 1 Gew.-% Triton X 100 in die Lösung gegeben und krätftig gerührt. Nach etwa 4 Stunden bei 4 bis 8 °C erfolgt eine weitere Zugabe von 5 Gew.-% Rizinusöl. Es wird dann eine Ölextraktion bei 15 °C vorgenommen. Die entstehenden Phasen werden getrennt und es wird anschließend mit einem Cuno-Schichtenfilter filtriert. Danach wird die Lösung auf eine C 18-Säule gegeben, die mit octadecylderivatisierten Stoffen beladen ist. Die Lösung wird dann auf pH 4 eingestellt und 100 g/l Maltose zugegeben. Nachfolgend wird eine Sterilfiltration vorgenommen und die sterilfiltrierte Lösung zwischen 22 und 24 Stunden bei 37 °C aufbewahrt. Die anschließend klare Lösung wird auf einen pH-Wert von 5,3 mittels 0,1 normaler Natronlauge eingestellt. Es erfolgt wiederum eine Ultra- und eine Diafiltration, danach eine Maltosezugabe von 100 g/l und die Einstellung der Lösung auf einen Proteingehalt von 50 g/l. Nach der folgenden Sterilfiltration wird die Lösung in 50 ml Infusionsflaschen, die sterilisiert und silikonisiert sind, abgefüllt, mit Stopfen verschlossen und verbördelt.

Patentansprüche

1. Verfahren zur Herstellung von zur intravenösen Applikation geeigneten virusinaktivierten Immunglobulinlösungen, dadurch gekennzeichnet, daß das Immunglobulin mit nichtionischen Tensiden behandelt wird, die anschließend durch Festphasenextraktion an hydrophoben Materialien entfernt werden.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß der pH-Wert der Lösung 5,0 bis 5,5 beträgt.

3. Verfahren nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß als nichtionische Tenside TNBP und/oder Triton X 100 verwendet werden.

4. Verfahren nach den Ansprüchen 1 bis 3, dadurch gekennzeichnet, daß nach der Behandlung mit nichtionischen Tensiden mit biologisch kompatiblen Pflanzenölen extrahiert wird und diese dann abgetrennt werden.

5. Verfahren nach Anspruch 4, dadurch gekennzeichnet, daß als Pflanzenöle Rizinusöl und/oder Sojaöl verwendet werden.

6. Verfahren nach den Ansprüchen 1 bis 5, dadurch gekennzeichnet, daß die Festphasenextraktion mit octadecylderivatisierten Stoffen, die auch für die Umkehrphasenchromatographie verwendet werden, durchgeführt wird.

7. Verfahren nach den Ansprüchen 1 bis 6, dadurch gekennzeichnet, daß die Festphasenextraktion durch C 18 Umkehrphasenchromatographie erfolgt.

8. Verfahren nach den Ansprüchen 1 bis 7, dadurch gekennzeichnet, daß der Lösung nach der Festphasenextraktion ein Disaccharid zugegeben wird.

9. Verfahren nach Ansprüchen 1 bis 8, dadurch gekennzeichnet, daß die fertige Lösung ein- oder mehrmals sterilfiltriert wird.

10. Verfahren nach den Ansprüchen 1 bis 9, dadurch gekennzeichnet, daß die Immunglobulinlösung vor der Virusinaktivierung und/oder nach der Sterilfiltration einer Ultra- und Diafiltration unterzogen wird.

11. Verfahren nach den Ansprüchen 1 bis 10, dadurch gekennzeichnet, daß die nach der Festphasenextraktion oder der Disaccharidzugabe eine Stabilitätsprüfung der hergestellten Lösung vorgenommen wird.

Europäisches Patentamt

EUROPÄISCHER RECHERCHENBERICHT

Nummer der Anmeldung
EP 92 11 1947

EINSCHLÄGIGE DOKUMENTE

Kategorie	Kennzeichnung des Dokuments mit Angabe, soweit erforderlich, der maßgeblichen Teile	Betrifft Anspruch	KLASSIFIKATION DER ANMELDUNG (Int. Cl.5)
X	EP-A-0 366 946 (NEW YORK BLOOD CENTER) * das ganze Dokument *	1-3,6-11	A61K39/395 A61L2/00
Y		4-5	C07K3/28
Y	EP-A-0 239 859 (NEW YORK BLOOD CENTER) * das ganze Dokument *	4-5	
A	EP-A-0 322 786 (NEW YORK BLOOD CENTER) * das ganze Dokument *	1-11	
A	EP-A-0 131 740 (NEW YORK BLOOD CENTER) * das ganze Dokument *	1-11	

RECHERCHIERTE SACHGEBIETE (Int. Cl.5)

A61L
C07K
A61K

Der vorliegende Recherchenbericht wurde für alle Patentansprüche erstellt

Recherchenort	Abschlußdatum der Recherche	Prüfer
DEN HAAG	16 NOVEMBER 1992	FERNANDEZ Y BRA F.

KATEGORIE DER GENANNTEN DOKUMENTE

X : von besonderer Bedeutung allein betrachtet
Y : von besonderer Bedeutung in Verbindung mit einer anderen Veröffentlichung derselben Kategorie
A : technologischer Hintergrund
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D : in der Anmeldung angeführtes Dokument
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& : Mitglied der gleichen Patentfamilie, übereinstimmendes Dokument

EPO FORM 1503 03.82 (P0403)

US005648472A

United States Patent [19]

Gebringer et al.

[11] Patent Number: 5,648,472

[45] Date of Patent: Jul. 15, 1997

[54] **PROCESS FOR PREPARING VIRUS-INACTIVATED IMMUNOGLOBULIN SOLUTIONS**

[75] Inventors: Werner Gebringer, Vienna, Austria; Patrick Selosse, Bordeaux, France

[73] Assignee: Octapharma AG, Glarus, Switzerland

[21] Appl. No.: 329,684

[22] Filed: Oct. 26, 1994

Related U.S. Application Data

[63] Continuation of Ser. No. 923,446, Aug. 3, 1992, abandoned.

[30] Foreign Application Priority Data

Aug. 2, 1991 [DE] Germany 41 25 625.5

[51] Int. Cl.[6] C07K 16/00; C07K 1/14; C07K 1/20

[52] U.S. Cl. 530/412; 530/415; 530/416; 530/389.1; 530/390.1

[58] Field of Search 530/412, 415, 530/416, 389.1, 390.1

[56] References Cited

U.S. PATENT DOCUMENTS

4,789,545 12/1988 Woods et al 424/101

5,094,960 3/1992 Bonomo 436/178

FOREIGN PATENT DOCUMENTS

0 058 993	9/1982	European Pat. Off.
0073571	3/1983	European Pat. Off.
0 099 445	2/1984	European Pat. Off.
0 131 740	1/1985	European Pat. Off.
0 239 859	10/1987	European Pat. Off.

OTHER PUBLICATIONS

Prince et al., PNAS, USA, 85:6944, 1988, Failure. HIV.

Primary Examiner—Frank C. Eisenschenk
Attorney, Agent, or Firm—Jacobson, Price, Holman & Stern, PLLC

[57] ABSTRACT

This invention describes a process for preparing virus-inactivated immunoglobulin solutions suitable for intravenous application, characterized in that the immunoglobulin is treated with non-ionic surfactants which subsequently are removed by solid-phase extraction on hydrophobic materials.

9 Claims, No Drawings

PROCESS FOR PREPARING VIRUS-INACTIVATED IMMUNOGLOBULIN SOLUTIONS

This is a continuation of application Ser. No. 07/923,446, filed Aug. 3, 1992, now abandoned, which is abandoned upon the filing hereof.

This invention is directed to a process for preparing virus-inactivated immunoglobulin solutions suitable for intravenous injection.

Immunoglobulins are humoral glycoproteins which, in electrophoresis of plasma or serum proteins migrate with the so-called γ-fraction and, thus, were formerly referred to as γ-globulins.

Because of their high antibody content immunoglobulins are used in the prophylaxis and therapy of infections.

It is known to prepare immunoglobulins for both intramuscular and subcutaneous application. A frequently employed method of preparation is the so-called Cohn-Oncley fractionation, also referred to as 6/9 method [Cohn et al. J. Am. Chem. Soc. 68, 459 (1946); Oncley et al. J. Am. Chem. Soc. 71, 541 (1949)].

However, this method of preparation is disadvantageous in that it results in a highly viscous solution which is applicable only by intra-muscular and subcutaneous routes and has high antibody concentrations in relatively small volumes

While the obtained product is stable at 4° C., proteolysis caused by plasmin contaminations may occur, however. Furthermore, IgA and IgG dimers may be present which, on application, may give rise to anaphylactic reactions in patients (Ullmann, encyclopedia of Industrial Chemistry 1989, A14, pp. 93, 94).

For this reason, intravenously applicable immunoglobulins have been developed which exhibit improved tolerance in the patient. They are prepared from the so-called fraction 3, or Cohn fraction 2 (Cohn et al., see above) at pH 4 using polyethyleneglycol, subsequent ethanol precipitation, ultrafiltration or diafiltration, and ion exchange chromatography. The immunoglobulin thus obtained is stabilized with mono- or disaccharides.

Such an improved process is described in EP 0,073,371.

Starting out from fraction 3 (Cohn et al., see above), ultrafiltration and diafiltration are conducted after dissolving and adjusting the pH value to pH 4. Subsequently, the filtrate obtained is concentrated to a protein content of 5% by weight, and the alcohol content is reduced to 8% by weight. Once the immunoglobulin solution thus obtained has been concentrated up to a protein content of 8%, a clear water-like solution having an ionic strength of 0.01 and a pH value of 4.2 is obtained. Using 10% by weight of maltose, the tonicity of the solution is adjusted at a protein content of 5% by weight. Subsequently, sterile filtration and lyophilization are effected. Prior to injection, the lyophilized material is dissolved in suitable media.

One major drawback of the immunoglobulins thus prepared for intravenous application is that prior to use, they must be dissolved in suitable media and can be stored in lyophilized form only.

Another drawback lies in the fact that in applying these immunoglobulins, viruses may be transferred to the patient since no virus inactivation occurs during the production process. Thus, hepatitis diseases and HIV infections have been reported after intravenous immunoglobulin administration (Ullmann's Encyclopedia of Ind. Chem., Vol. A14, 1989, pp. 102, 103).

Therefore, the technical problem of the invention was to develop a process for virus inactivation of intravenously

injectable immunoglobulins resulting in a product wherein virus transfer to the patient during application does not occur, and which is stable to an extent that even without lyophilization, it can be prepared and stored directly as an injectable solution

The technical problem of the invention is solved by a process, characterized in that the immunoglobulin is treated with non-ionic surfactants which subsequently are removed by solid-phase extraction on hydrophobic materials

As the non-ionic surfactants, especially TNBP and/or TRITON×100 are used. Preferably, the pH value of the solution is from 5.0 to 5.5.

In a preferred embodiment, subsequent to treatment with non-ionic surfactants, an extraction is conducted using biologically compatible vegetable oils followed by removal thereof. As the vegetable oils, castor oil or soy bean oils are used preferably.

The subsequent solid-phase extraction is conducted in preferred fashion using octadecyl-derivatized materials also used in reversed-phase chromatography. In a particularly preferred embodiment, solid-phase extraction is effected by reversed-phase chromatography on octadecyl (C-18) resin.

Following solid-phase extraction, a disaccharide may be added to the final product for stabilization. The final solution then is subjected to single or multiple sterile filtration.

Furthermore, the immunoglobulin solution may be subjected to ultra- or diafiltration prior to virus inactivation and/or subsequent to sterile filtration.

In the following, the production process of the invention is described in detail. Initially, as is known from prior art, the so-called Cohn fraction II is dissolved in water until a completely clear solution is obtained. Then, the solution is adjusted to a pH value of from 4.0 to 5.0, preferably 4.5, and filtered to remove contaminations.

Subsequently, the solution is subjected to ultrafiltration, and the solution is pre-concentrated. The ultrafiltration exclusion limit is 30,000 Dalton. In this step, contaminations having low molecular weights are removed especially. Subsequently, a diafiltration is effected to remove ions, which is followed by the actual virus inactivation

In doing this, the solution first is cooled down to from 4° to 8° C., and the pH value is adjusted to from 5.0 to 5.5, preferably 5.3. Then, non-ionic surfactants, preferably TNBP and/or Triton×100 are added, and this solution then is stirred for several hours. Subsequently, in a preferred embodiment, a vegetable oil extraction may be carried out. Here, 5% by weight of vegetable oil is added to the solution, the solution then is brought to room temperature and mixed with the vegetable oil by stirring. The subsequent phase separation is followed by filtration.

Then, the solution is applied to a C 18 column and is subjected to chromatography. Subsequent to chromatography, the pH value is adjusted to pH 4. Maltose is added to adjust tonicity. Subsequent to the following sterile filtration, stability of the solution thus obtained is tested by storing at 37° C. for at least 22 hours. If the solution shows turbidity, it cannot be used. If the solution does not show turbidity within this period of time, the pH value is adjusted to from 5.0 to 5.5, preferably 5.3, further ultrafiltration and diafiltration are carried out, and the solution thus obtained is adjusted to a protein content of 50 g/l by addition of maltose. Subsequently, another sterile filtration is carried out, and the solution is directly filled into infusion bottles.

The product thus obtained may be used for direct intravenous injection and is free of viruses.

The following embodiment is given to explain the process of the invention in more detail.

EXAMPLE

The Cohn fraction II is dissolved using a sixfold amount of water and stirred until a clear solution is obtained. Subsequently, the pH value is adjusted to 4.5 using 0.5N HCl. Next, this is followed by ultrafiltration wherein the solution is pre-concentrated to 90 g/l. A Novasette 30K membrane type is used. Subsequently, dilution is effected using a fivefold amount of water, and diafiltration is carried out at from 0.3 to 0.5 bars. Following this diafiltration, the diafiltered solution is adjusted to a protein content of 70 g/l. The solution is cooled down to 4° to 8° C. and adjusted to a pH value of 5.3, using 0.1N sodium hydroxide solution. Subsequently, 0.3% by weight of TNBP and 1% by weight of TRITON×100 (non-ionic surfactant) are added to the solution, followed by vigorous stirring. After about 4 hours at 4° to 8° C., 5% by weight of castor oil is added. Then, oil extraction is conducted at 15° C. The resulting phases are separated, followed by filtration using a Cuno sheet filter. Subsequently, the solution is applied to a C 18 column charged with octadecyl-derivatized materials. Then, the solution is adjusted to pH 4, and 100 g/l of maltose is added. This is followed by sterile filtration, and the sterile-filtered solution is stored at 37° C. for between 22 and 24 hours. The solution, being clear then, is adjusted to a pH value of 5.3 using 0.1N sodium hydroxide solution. Again, an ultrafiltration and a diafiltration are carried out followed by addition of maltose of 100 g/l and adjusting the solution to a protein content of 50 g/l. Subsequent to the following sterile filtration, the solution is filled into sterilized and siliconized 50 ml infusion bottles which are sealed with a stopper and tied up.

We claim:

1. A process for preparing an envelope virus-inactivated immunoglobulin solution suitable for intravenous application, comprising treating the immunoglobulin with TNBP and/or TRITON×100, followed by an extraction using biologically compatible vegetable oil, which TNBP and/or TRITON×100 and vegetable oil are subsequently removed by solid-phase extraction on hydrophobic materials.

2. The process according to claim 1, wherein the pH value of the solution is from 5.0 to 5.5.

3. The process according to claim 2, wherein castor oil and/or soy bean oil are used as vegetable oil.

4. The process according to claim 1, wherein the solid-phase extraction is carried out using octadecyl-derivatized materials also used for reverse-phase chromatography.

5. The process according to claim 1, wherein the solid-phase extraction is carried out using C 18 reversed-phase chromatography.

6. The process according to claim 1, wherein subsequent to solid-phase extraction, a disaccharide is added to the solution.

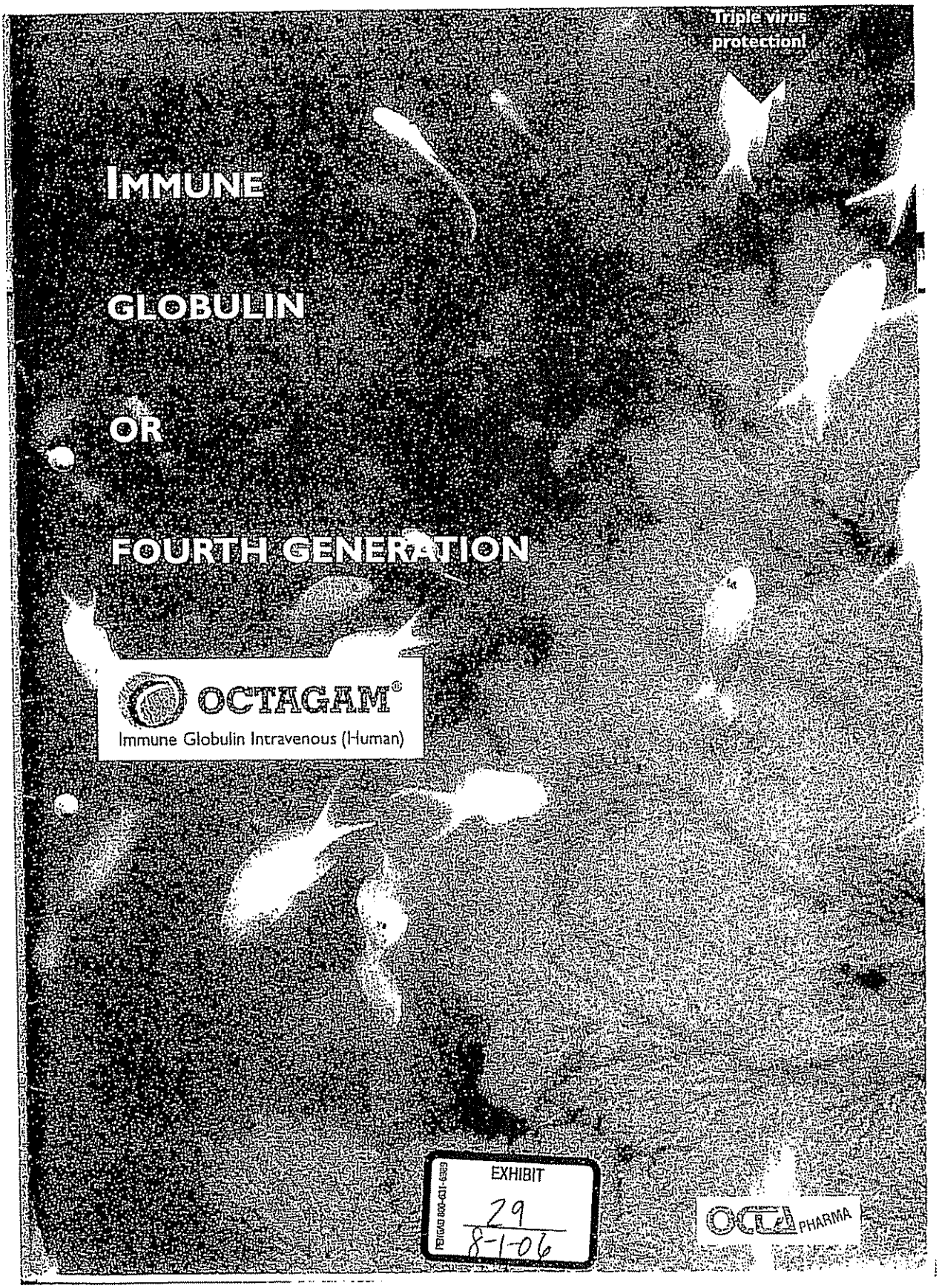
7. The process according to claim 1, wherein the final solution is subjected to a single or multiple sterile filtration.

8. The process according to claim 1, wherein the immunoglobulin solution is subjected to ultrafiltration and diafiltration prior to virus inactivation and/or subsequent to sterile filtration.

9. The process according to claim 1, wherein subsequent to solid-phase extraction or disaccharide addition, a stability test of the prepared solution is conducted.

* * * * *

EXHIBIT 17

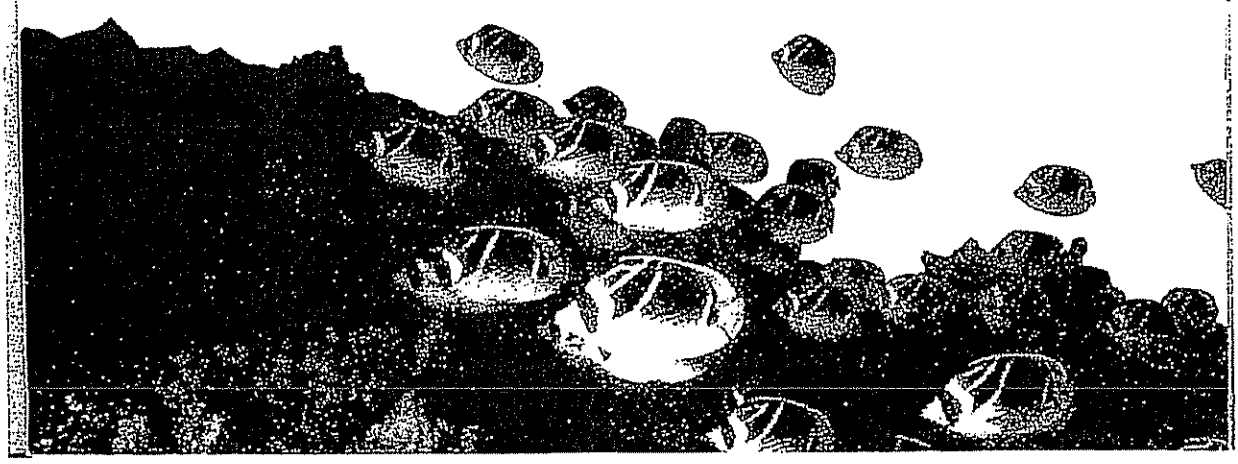

Triple virus protection!
IMMUNE
GLOBULIN
OR
FOURTH GENERATION
OCTAGAM®
Immune Globulin Intravenous (Human)
EXHIBIT
29
8-1-06
PHARMA

2

AN OCEAN FULL OF LIFE

The ocean is magnificent and intriguing. Within its depths is found the fascination of all life - beauty, intrigue, and the struggle to survive. The ocean's ecosystem is constantly challenged. Life within it will endure only if the challenges of internal and external threats are successfully met. But it is a perpetual struggle. Like the ocean's ecosystem, the human immune system is mysterious and fascinating in its response to perpetual challenges. The response is characterized by a coordination of many single elements: a ceaseless flow of information and a multitude of reactions and adaptations. Questions, answers, and verification are closely intertwined in the struggle for survival. Like the life in the ocean, the human immune system has evolved through constant effort to meet the internal and external challenges presented. Similar to life in the ocean, form follows function.

Not all immune globulins are created equal!



A NEW GENERATION OF QUALITY

The 4th generation of immune globulins is a new generation: a new generation of quality, - but even more: a new generation of responsibility.

EFFICACY

SAFETY

CONVENIENCE

1st Generation:
chemically or enzymatically altered (impaired F_c function)

2nd Generation:
Complete F_c function

3rd Generation:
Complete F_c function high purity, liquid formulation

4th Generation:
Complete F_c function high purity, stable at room temperature, liquid, triple viral protection

PRODUCT WITH CHARACTER

OCTAGAM® is a fourth generation immune globulin exhibiting the following important characteristics:

OCTAGAM® CHARACTERISTICS:

EFFICACY

- Native molecules, no enzymatical or chemical treatment
- Intact F_c function
- Over 97% monomer and dimer content
- IgG subclass distribution as in normal plasma
- Over 99% IgG
- Mean half-life 28 days

SAFETY

- Low anticomplementary activity
- Low isoagglutinin titres
- Low prekallikrein activator
- Trace presence of IgA
- Normal osmolality
- **VIRUS SAFETY** through Cohn-Fractionation, SD-inactivation and pH4-treatment

CONVENIENCE

- Liquid, ready to use
- Room temperature storage
- Shelf-life of 2 years

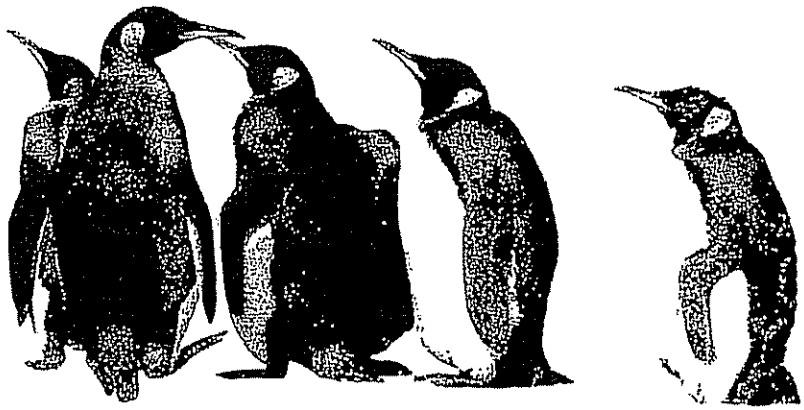
A ROOM FULL OF REFRIGERATORS?

You are undoubtedly familiar with this storage problem: Many immune globulins have to be refrigerated or they have to be dissolved by means of time consuming procedures - before they are brought to room temperature for use
Not **OCTAGAM**[®]:
OCTAGAM[®] is the IGIV you can store for two years at room temperatures up to 25°C. Simply put it on the shelf:
It will be available:

WHEN YOU NEED IT •
WHERE YOU NEED IT •
THE QUANTITY YOU NEED •
READY FOR IMMEDIATE •
INTRAVENOUS ADMINISTRATION

CONVENIENCE

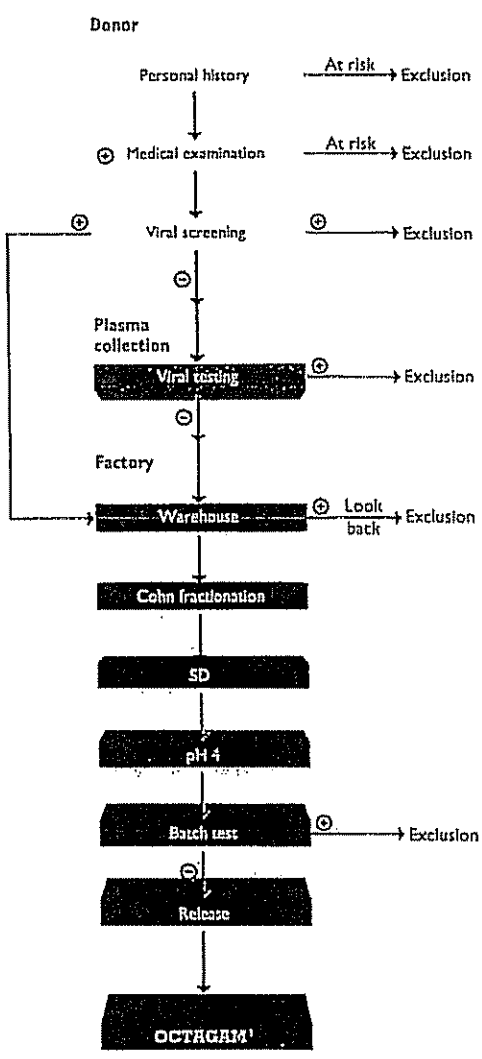
KEEP OCTAGAM® IN STOCK AND FORGET ABOUT YOUR REFRIGERATOR!



6

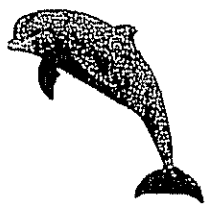
TRIPLE PROTECTION

Today, the crucial question to everything in biopharmaceutical therapy is: Viral safety?



OCTAGAM® offers an immune globulin product with triple protection to help ensure virological safety.

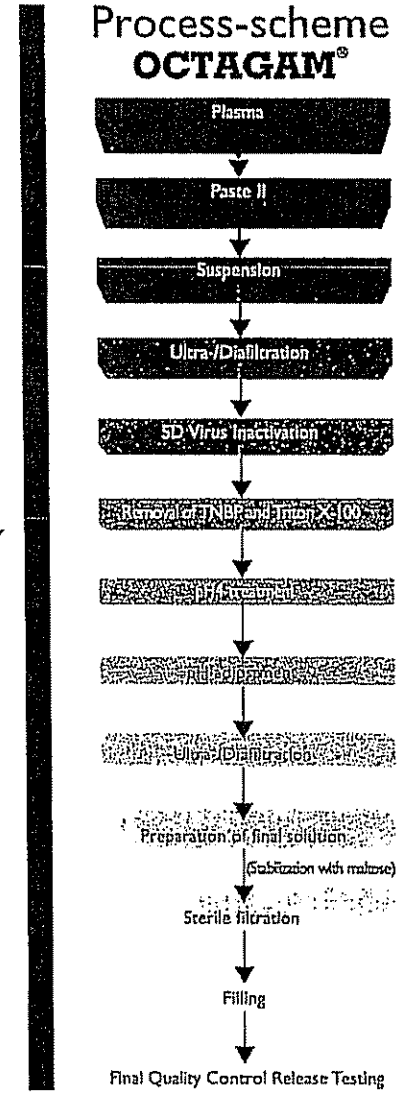
1. **Viral reduction by Cohn-Fractionation**
2. **SD-method to eliminate encapsulated viruses**
3. **pH4-treatment to destroy non-encapsulated viruses**



OCTAGAM® SAFETY: NO COMPROMISE

The manufacturing process of **OCTAGAM®** means uncompromised safety standards

SAFETY



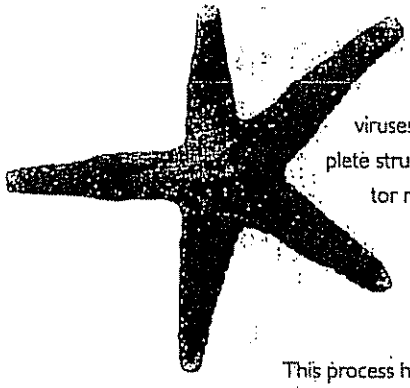
METHODS OF VIRAL INACTIVAT

Viruses can be transmitted by blood and blood products. Here, we try to briefly discuss the many types of methods employed with viral reduction for plasma derived products

The following information is extrapolated from the bibliography on page 7

1. Cohn-Fractionation:

The principal precipitation agent in Cohn-Fractionation is ethanol The concentration ranges from 8% - 40% The subzero temperatures during the Cohn-Fractionation further reduce the influence of ethanol, but the long processing times combined with pH and filtration improve the effect on viral reduction Additionally the type of partitioning during this process is important as a first step in viral transmission Recent HCV-transmission have shown that Cohn-Fractionation is insufficient



2. Solvent/Detergent:

This organic mixture disrupts the membranes of viruses that have lipid envelopes The result is the complete structural disruption or destruction of the cell receptor recognition site In both cases the viruses become non infectious This is demonstrated with **OCTAGAM**™

3. pH4-treatment:

This process has demonstrated significant efficacy in destruction or inactivation of nonlipid-coated viruses and some lipid-coated viruses This is proven in the data shown for **OCTAGAM**™

ON FOR IMMUNE GLOBULINS

4. Chromatographic methods:

Most of the chromatographic processes lead only to a certain distribution of the viruses. With these methods it is essential to prevent contamination by applying effective virus inactivation treatments to the matrixes. Each chromatographic method should include at least one specific step for inactivating viruses.

5. Heating in solution (pasteurization):

This method has been used since 1948 for albumin products: heating at least 10 hours at 60°C. However, most proteins when heated at this temperature in the liquid state are denatured. Therefore, additional stabilizers such as amino acids, citrate, or sugars must be used for protein stabilization.

6. Heating of dry products:

When freeze-dryed, most proteins tolerate temperatures up to 68° C. However, both the stability of the proteins and the degree of virus inactivation depend on the moisture content and the type and quality of the stabilizers used in addition to the method of freeze-drying. A temperature of 68°C is sufficiently high to inactivate HIV and lowers the risk of NANBV and HBV.

*In general, Cohn fractionation and/or chromatography methods combined with heat treatment methods or pH-treatment reduce the risk of nonlipid-coated viruses and solvent/detergent use eliminates lipid-coated viruses. It is important to question manufacturers about the methods employed to reduce **both** lipid-coated and non lipid-coated viruses.*

Specific virus inactivation procedures should not result in unacceptable alterations to the biochemical and immunological properties of the immune globulin molecule. This is of great importance because biochemically altered immune globulin molecules are liable to have reduced in vivo half-lives due to a reduced half life or reduced effector functions such as opsonisation, complement activity, or Fc function.

References:

Surmeta H. Inactivation of Viruses in Blood and Plasma Products. Transfusion Medicine Reviews. 7:42-57, 1993.

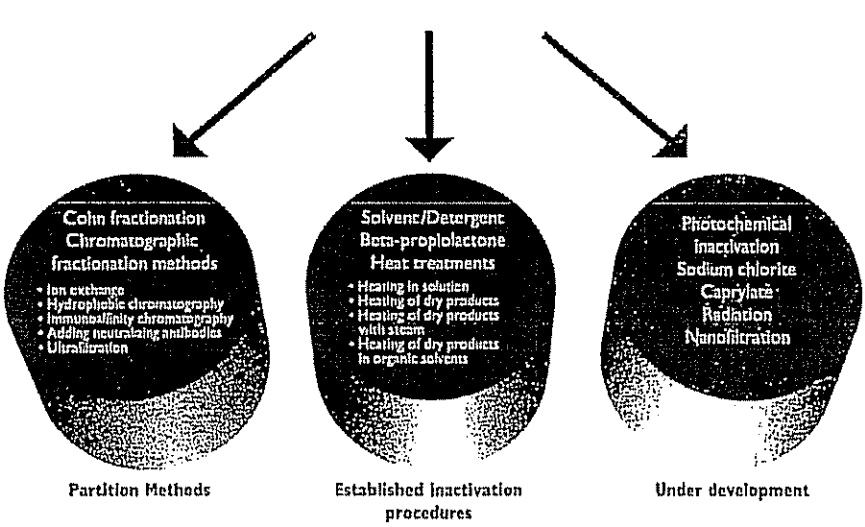
Cuthbertson B, Reid RG, Foster PR. Viral Contamination of Human Plasma and Procedures for Preventing Virus Transmission by Plasma Products. Blood Separation and Plasma Fractionation. Wiley-Liss, Inc. 1991, pp. 105-135.

Nielsen H. Immunoglobulin preparations for intravenous and subcutaneous use. Allergy 49:69, 1994.

Bieson J. et al. Virus reduction of a new polyvalent intravenous immunoglobulin (OCTAGAM®). Vox Sanguinis 67 suppl. 3:161, 1994.

Schneider L. et al. Outbreaks of Hepatitis C associated with intravenous immunoglobulin administration. United States, October 1993 - June 1994. Morbidity and Mortality weekly report. 43(49):905-906, 1994.

METHODS USED FOR VIRAL INACTIVATION/REDUCTION IN PLASMA PRODUCTS



It is necessary to prove that the manufacturing method ensures a titre reduction by at least the factor 10^{-7} for enveloped viruses through steps designed to inactivate and eliminate viruses. In the case of non-enveloped viruses a titre reduction of at least 10^{-4} must be documented. Manufacturing methods must include two steps which, in the case of enveloped viruses, produce a titre reduction of the order of magnitude of 10^{-4} in each case. In the case of non-enveloped viruses it is necessary to prove at least a corresponding step with this order of magnitude.

Notice relating to measures against drug-associated risks. Reduction of the risk of transmission of hematogenous viruses in the case of drugs manufactured by fractionation of plasma of human origin.

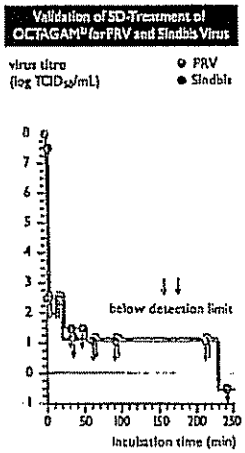
11th August 1994. Excerpted from »Bundesanzeiger« 26th August 1994. Translated by Octapharma GmbH

OCTAGAM®-SAFETY AT FIRST SIGHT

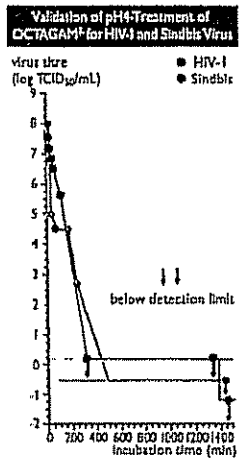
Reduction of viruses during manufacturing

Step	Virus	Reduction (log)
Cold Fractionation	HIV-1	>5.00
	Sindbis	>5.00
	Pseudorabies (PRV)	>7.26
	Poliovirus	>3.80
	Coxsackie B6	4.70
	SV 40	>5.00
Solvent/Detergent	HIV-1	>5.00
	Pseudorabies	>5.00
	Sindbis	>5.00
	SV 40	>5.00
Pasteurization	HIV-1	>8.30
	Sindbis	>8.20
	Pseudorabies	>7.00
	SV 40	>7.00
Nanofiltration	Coxsackie B6	>5.00
Total Reduction	HIV-1	>19.35
	Sindbis	>23.10
	Coxsackie B6	8.48
	Pseudorabies	>21.60
	SV 40	> 6.66

SD-treatment



pH4-treatment



OCTAPHARMA

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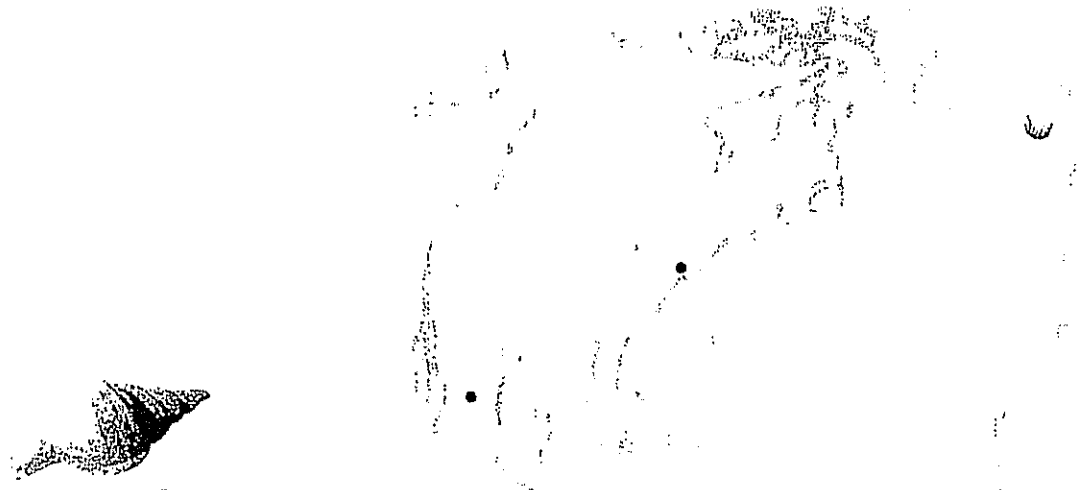

- *Headquarters*
  Ziegelbrücke S[illegible]
- *Production plants*
  Vienna, Austria and Tel A[illegible] Israel

  Sales Offices
  Austria, Belgium, Denmark, France, Germany, Great Britain, Ireland, Israel, Norway, Portugal, Russia, Sweden, Switzerland
- *Licencees:*
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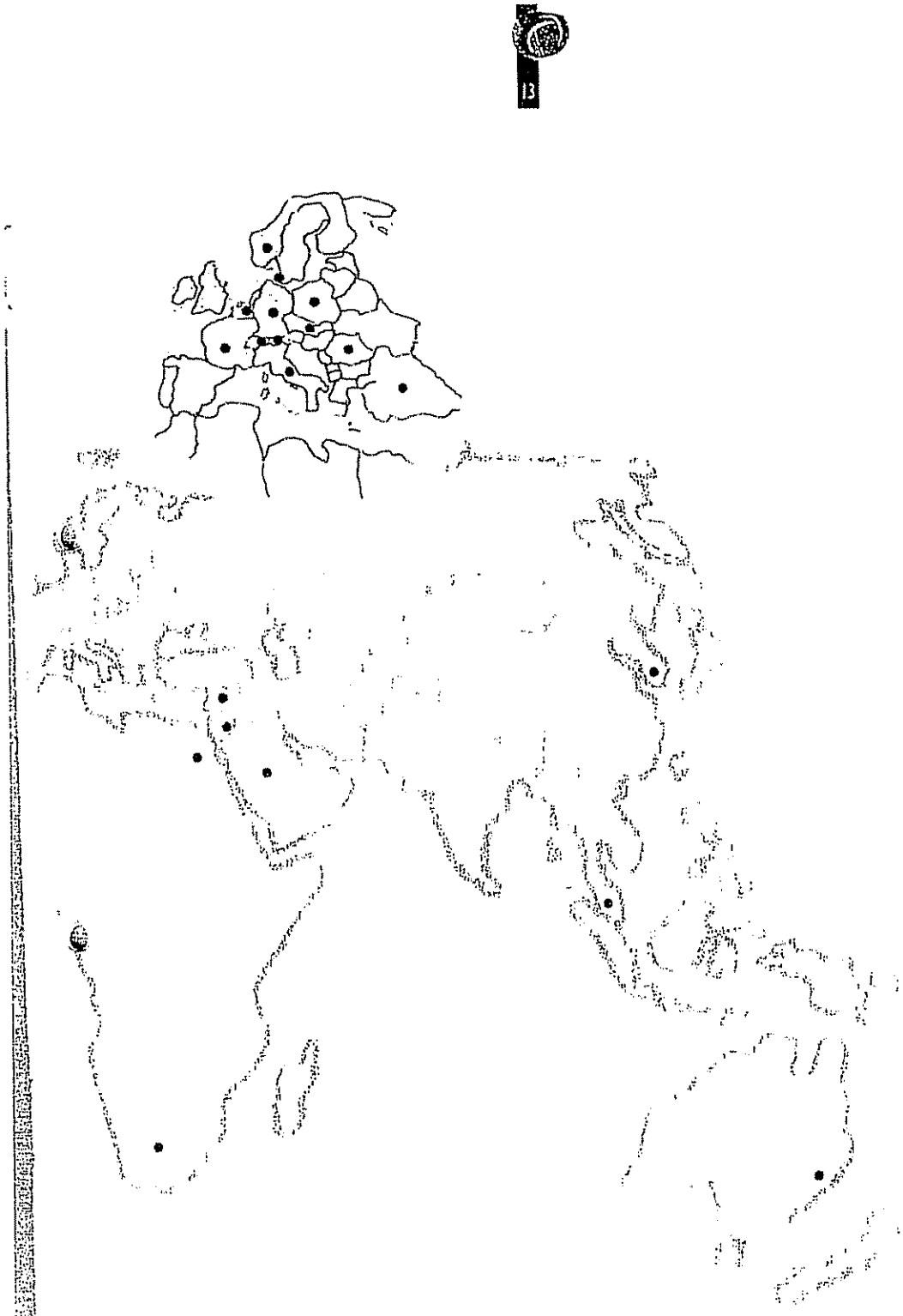
  Brazil, Egypt, Malaysia, Mexico, Poland, Romania, Slovenia, Slovakia, Arabia, Syria, Turkey, United Arab Emirates, Venezuela

  R&D
  Austria, Germany, Great Britain



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INSTRUCTIONS FOR USE - OCTAGAM®

Immune Globulin Intravenous Human

Active Ingredients

Presentation and Size of Packaging

Classification

Pharmaceutical Company Name and Address

Indications

Contraindications

Use during Pregnancy and Lactation

Special Considerations for Use

Effects on Motorists and Operators of Machinery

General Interactions and Drug Interactions

Dosage Instructions

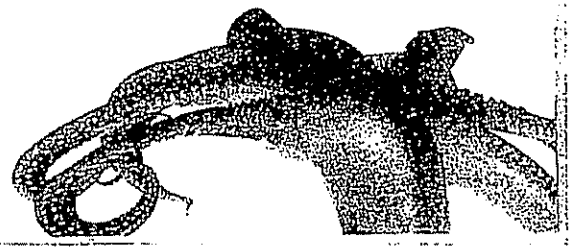
Methods and Administration

Overdosage

Adverse Events

Immediate Measures to be taken in Case of Intolerance Reactions

Clinical Symptoms	Measure

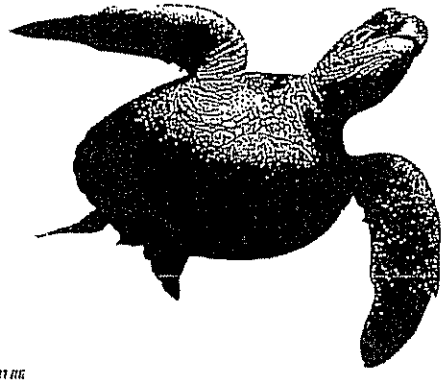


Storage and Stability

Note

Date of Information

Store all drugs out of the reach of children!



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LIQUID, READY TO USE

ROOM TEMPERATURE STORAGE

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Headquarters • SWITZERLAND • OCTAPHARMA [illegible] • Seidenstrasse 2 • Hauptsitz Park • CH-8853 Lachen SZ • Tel. [illegible]

EXHIBIT 18

US005410025A

United States Patent [19]

Möller et al.

[11] Patent Number: 5,410,025

[45] Date of Patent: Apr. 25, 1995

[54] UNMODIFIED INTRAVENOUSLY ADMINISTERED IMMUNOGLOBULIN PREPARATIONS CONTAINING IMMUNOGLOBULIN M AND/OR A

[75] Inventors: Wolfgang Möller, Oberursel; Detlef Piechaczek, Munster, both of Germany

[73] Assignee: Biotest Pharma GmbH, Dreieich, Germany

[21] Appl. No.: 154,149

[22] Filed: Nov. 18, 1993

Related U.S. Application Data

[63] Continuation of Ser. No. 782,747, Oct. 18, 1991, abandoned, which is a continuation of Ser. No. 561,037, Aug. 1, 1990, abandoned.

[30] Foreign Application Priority Data

Aug. 17, 1989 [DE] Germany 39 27 111.0

[51] Int. Cl.[6] A61K 35/14; C07K 3/12

[52] U.S. Cl. 530/390.5; 530/387.1; 530/412; 530/416

[58] Field of Search 530/389.1, 388, 369, 530/387.1, 412, 416, 390.5

[56] References Cited

U.S. PATENT DOCUMENTS

3,808,189	4/1974	Breuer .	
4,613,501	9/1986	Horowitz	424/89
4,877,865	10/1989	Radnick et al.	530/387
5,159,064	10/1992	Mitra et al.	530/388.1

OTHER PUBLICATIONS

Ion Exchange Chromatography, Principles and Methods.
Jawetz et al., Review of Medical Microbiology, p. 350, 1984.
Goding et al., Monoclonal Antibodies: Principles and Practice, pp. 112–116, 1986.
Kabat et al., Experimental Immunochemistry, p. 217, 1961.
Arzneimittel-Forschung Drug Research, 1985, pp. 2–11.
Pentaglobin, Human Immunoglobulin Containing Ig/VI for Intravenous Drug Use Against Bacterial Organisms
Pentaglobin, The Chance to Survive Septic Shock.
DMW 1987, 112.Jg., Nr. 33, 1267–1271
Intensivmedizin und Notfallmedizin 24 (6), 314 (1987).
Noack, et al., Intravenous immunoglobulin M in the treatment of infants with septicaemia and bacterial meningitis.
Thrombosis and Haemostasis, THHADQ 58(1) 1–676 (1987) Jul. 6, 1987.
2. Wiener Schockgespräche, May 11–14, 1988.
Journal of Obstetrics and Gynaecology (1989) 10. (Suppl 1) S 25 S26.
Abstracts, SY 82, Use of an IgM Enriched Intravenous Immunoglobulin in Bone Marrow Transplantation, C. H. Poynton, Cardiff, Wales.
Abstracts, Protection of infection-prone cardiopulmonary bypass patients by postoperative immunoglobulin prophylaxis, H. G. Kress, Wurzburg, FRG
Critical Care Medicine, 1991, vol. 19, No. 9, Treatment of Gram-negative septic shock with an immunoglobulin preparation.
W. Stephan, Investigations to Demonstrate the Antibacterial and Antitoxic Efficacy of an IgM-Enriched Intravenous Immunoglobulin Preparation; Immune Consequences of Trauma; 1989.
Hood et al., Immunology, 1984, pp. 84–87.
Johnstone et al., Immunochemistry in Practice, 1987. pp. 72–77.
Roitt et al., 1989, Immunology, p. 57, Fig. 5.18.
Cheo et al., T. Chromatography, vol. 444 pp. 153–164 (1988).

Primary Examiner—David L. Lacey
Assistant Examiner—F. C. Eisenschenk
Attorney, Agent, or Firm—Sprung Horn Kramer & Woods

[57] ABSTRACT

Intravenously administered chemically unmodified immunoglobulin preparation containing more than 5% of its total immunoglobulin by weight consisting of IgM and/or more than 10% of its total immunoglobulin of IgA and with a low anticomplementary activity, and method of preparing it by anion-exchange chromatography.

8 Claims, No Drawings

UNMODIFIED INTRAVENOUSLY ADMINISTERED IMMUNOGLOBULIN PREPARATIONS CONTAINING IMMUNOGLOBULIN M AND/OR A

This application is a continuation, of application Ser. No. 782,747, filed Sep. 18, 1991, now abandoned which is a continuation of application Ser. No. 561,037, filed Aug. 1, 1990, now abandoned.

The present invention relates to intravenously compatible immunoglobulin preparations that have not been chemically modified and that contain more than 5% immunoglobulin M (IgM) and/or more than 10% immunoglobulin A (IgA) in terms of their total immunoglobulin content.

The immunoglobulins are a group of glycoproteins that occur in the body in response to the appearance of foreign antigens and are responsible for attacking and eliminating them. Although there are several classes of immunoglobulins, only IgG, IgA, and IgM occur in the plasma in significant concentrations.

At approximately 12 mg/ml, IgG is the major immunoglobulin in the plasma. It is mainly responsible for combating viral infections. The concentration of IgM is definitely lower—approximately 1.5 mg/ml. IgM is mainly involved in combating bacterial pathogens and in picking up bacterial toxins. IgA has a mean plasma concentration of 3.5 mg/ml, has the responsibility of neutralizing various viruses, those of poliomyelitis, measles, and influenza for example, and is encountered in its secretory, dimeric form in the seromucous secretions.

Immunoglobulin preparations have been employed for passive immunotherapy for some 40 years. Most of these substances are pure IgG preparations, with only low levels of IgA and IgM. Immunoglobulin preparations, furthermore, could be administered only intramuscularly prior to the 1960's, and their painful side effects prevented the use of large doses.

Several intravenously applicable IgG preparations were subsequently developed either by modifying the immunoglobulin chemically or enzymatically or by other methods.

IgG preparations, however, that also contained significant levels of IgM and/or IgA—those described in German Patent 2 404 265 or U.S. Pat. No. 3 808 189 for example—were still applicable only intramuscularly.

The first and up to now only intravenously applicable immunoglobulins to contain IgM and/or IgA are described in European Patent 0 013 901 and in German OS 3 825 429. Both of these preparations are essentially rendered intravenously applicable by being chemically modified with β-propiolactone (BPL). One measure of the intravenous compatibility of immunoglobulin preparation is the anticomplementary activity (ACA) described by E. Kabat and M. Mayer in Experimental Immunochemistry, 2nd ed., 1964, Springfield, Ill., Thomas Brooks, 133–240.

The object of the present invention is to develop immunoglobulin preparations that contain IgM and/or IgA, that have a low anticomplementary activity, and that are intravenously applicable although not chemically modified.

This object is attained by treating an immunoglobulin solution that contains immunoglobulin M and/or immunoglobulin A with an anion exchanger, gradient-eluting a fraction with a low anticomplementary activity, and optionally subjecting the fraction to a brief treatment at low pH and/or high temperature.

It has been surprisingly discovered that anion-exchange chromatography will attach the fraction responsible for the high anticomplementary activity so securely that subsequent elution under appropriate condition will wash out approximately 95% of the immunoglobulins collected, including most if the IgM and IgA with a low anticomplementary activity.

If the starting material has an elevated anticomplementary activity, the additional brief treatment of the eluate at low pH and/or high temperature will lower it to a level that is normal for intravenously applicable products.

Also surprising was the discovery that using a starting material with a low anticomplementary activity will sometimes make it possible to lower the anticomplementary activity just by treating the eluate from the anion exchanger at a low pH and/or high temperature with no need to remove some of the IgA and IgM through chromatography.

It will be obvious that the starting material for the anion-exchange chromatography should be produced under conditions that will protect the protein as much as possible against denaturation. Appropriate starting materials are solutions that contain immunoglobulin—Cohn Fraction II/III or Cohn Fraction III, other plasma fractions that contain IgA or IgM, such other solutions as milk or milk fractions, other body fluids, or residues from cultures of cells that produce IgA and/or IgM for example.

A fraction—Cohn Fraction II/III or III for example—that contains immunoglobulin can for example be dissolved in a buffer, and most of the impurities eliminated by precipitation with 0.5 to 5% octanoic acid at a pH of 4 to 6 and preferably 5. The solution is then treated at a low conductivity with an anion exchanger, attaching most of the IgA and IgM. Adsorption can be carried out batchwise, in a chromatography column, or on membranes.

If the desired product is to contain IgA and IgM the elution is carried out with a salt gradient that will leave approximately 10 to 20% of the IgM on the matrix. The precise eluting conditions depend on the type of anion exchanger and range from 10 to 400 m_{eq}, depending on the matrix and pH. The specifications for specific matrices are cited in the examples.

If the product is intended to contain IgA and not IgM, the elution is carried out at a lower osmolarity and the IgM will remain adsorbed onto the matrix. Depending on the chromatographic conditions, the eluate will contain 30 to 60% IgA and 70 to 40% IgG. The IgA can be further purified by appropriate measures.

If on the other hand the eluate also contains significant levels of IgM, the anticomplementary activity can be further decreased by 1 minute to 24 hours of additional treatment at a low pH, preferably 4 to 4.5, and/or at a higher temperature, 40° to 60° C. and preferably 50° to 54° C.

A pure IgM solution, extensively free of IgA, can be obtained by washing the anion exchanger ahead of time with a buffer to elute the IgA before the IgM.

When the anticomplementary activity in the starting material is lower, all of the IgM fraction that is adsorbed onto the anion exchanger can sometimes be eluted. In this situation, treating the eluate for 1 minute to 4 hours at a low pH, preferably 4 to 4.5, and/or at a high temperature, 40° to 60° C. and preferably 50° to 54°

C., will be sufficient by itself to reduce the activity to a tolerable level.

The solution can then be concentrated by ultrafiltration and its electrolyte level adjusted to that of the final intravenous formulation by diafiltration. The anticomplementary activity of the final product will then be in the range conventional for common commercial intravenous IgG preparations or for the chemically modified IgM preparation Pentaglobin.

Since the anticomplementary activity of the IgG fraction not attached to the anion exchanger is also very low, the fraction can be employed in conjunction with the fractions that contain IgA and/or IgM in accordance with the invention to prepare mixtures that can be converted into intravenously compatible immunoglobulin preparations with a low anticomplementary activity and a desired ratio of IgG, IgA, and IgM. An immunoglobulin preparation that contains IgM and IgA and has the same composition as the commercial, chemically modified Pentaglobin—80% IgG, 10% IgA, and 10% IgM—for example but with an anticomplementary activity that is equal or lower can be prepared.

The immunoglobulin preparations that contain IgM and/or IgA in accordance with the invention can be subjected before or after the steps of the method in accordance with the invention to such in-themselves known sterilization procedures as treatment with β-propiolactone and ultraviolet light, treatment with solvents and/or detergents, or pasteurization.

The invention will now be described with reference to examples without being limited to them.

EXAMPLE 1

10 kg of Cohn paste III were dissolved in 50 l of 0.1M acetate buffer at a pH of 5 and treated with 1.5 kg of octanoic acid. The precipitate was centrifuged out after 4 hours and the supernatant dialyzed against 20 mM of piperazine and 20 mM of sodium chloride at a pH of 6. The solution is then applied onto a 5-l column of TMAE-Fraktogel (Merck, Darmstadt) equilibrated with the same buffer and chromatographed in 5 runs. The unattached IgG fraction was collected and concentrated by ultrafiltration.

An IgA-rich fraction was eluted with 20 mM of piperazine and 100 mM of sodium chloride at a pH of 6 and an IgM-rich fraction with 20 mM of piperazine and 150 mM of sodium chloride at a pH of 6. The rest of the attached protein was then washed out of the column with 20 mM of piperazine and 190 mM of sodium chloride at a pH of 6.

Table 1 shows the fraction's composition and anticomplementary activity.

TABLE 1

	IgG g/l	IgA g/l	IgM g/l	ACA CH 50/ml
IgG fraction	50	0.3	0	9
IgA fraction	31	18	0.3	17
IgM fraction	9	9	31	41
residual fraction	8	3	39	441

A chemically unmodified immunoglobulin preparation comprising 80% IgG, 10% IgA, and 10% IgM was then mixed from the IgG, IgA, and IgM fractions in this example and compared with the commercial product Pentaglobin, which is modified with β-propiolactone (Table 2).

TABLE 2

	IgG g/l	IgA g/l	IgM g/l	ACA CH 50/ml
Inventive preparation:	40.0	4.9	5.0	13
Pentaglobin, Batch 1462019 (reference):	43.4	4.2	5.0	26

EXAMPLE 2

1 kg of Cohn Paste III was treated as described in Example 1. It was applied onto a chromatography column and immediately eluted with 20 mM of piperazine and 160 mM of sodium chloride at a pH of 6. The fraction contained 50% IgG, 23% IgA, and 27% IgM in terms of the overall immunoglobulin content. The anticomplementary activity was 26 CH 50/ml.

EXAMPLE 3

1 kg of Cohn Paste III was treated as described in Example 1 and passed in two runs through a 2 l column of QMA-Accell. It was immediately eluted with 20 mM of piperazine and 20 mM of sodium chloride at a pH of 4.7. The fraction contained 38% IgG, 27% IgA, and 35% IgM in terms of the overall immunoglobulin content. The anticomplementary activity was CH 50/ml and decreased to 20 CH 50/ml subsequent to 30 minutes of treatment at a pH of 4.0.

EXAMPLE 4

10 kg of Cohn Paste II/III were treated as described in Example 1. Since Paste II/III contains more IgG than Paste III does, the level of octanoic acid was decreased to 0.75 kg. Table 3 illustrates the properties of the eluates.

TABLE 3

	IgG g/l	IgA g/l	IgM g/l	ACA CH 50/ml
IgG fraction	49	1	0	4
IgA fraction	15	15	0.2	7
IgM fraction	9	10	3.0	19
Remaining fraction	10	4	36	193

EXAMPLE 5

1 kg of Cohn Paste III was treated as described in Example 1. It was applied onto the chromatography column, washed with 20 of piperazine and 120 mM of sodium chloride at a pH of 6, and eluted with 20 mM of piperazine and 175 mM of sodium chloride at a pH of 6. The eluate was concentrated by ultrafiltration and adjusted by diafiltration to a protein level of 50 g/l in 75 mM of sodium chloride and 2.5% glucose at a pH of 7. Table 4 illustrates the preparation's properties.

TABLE 4

	Protein g/l	IgM g/l	ACA CH 50/ml	Rel. Titer[2] Ps. aerug.
IgM preparation (invention)				
After chromatography	54.3	50.5	2300	40 960
After 20 min. @ 50° C.	54.3	50.5	29	40 960
After freeze-drying[1]	50.7	46.5	27	40 960
Pentaglobin				
Batch 1462019	50.3	5.0	26	1 280

TABLE 4-continued

	Protein g/l	IgM g/l	ACA CH 50/ml	Rec. Titer[2] *Ps. aerug.*
(reference)				

[1]Reconstituted with distilled water.
[2]The antibacterial titer against *Pseudomonas aeruginosa* was determined by passive hemagglutination as described by E. Hass, Bact. Rev. 20, 166 (1956).

EXAMPLE 6

6 kg of Cohn Paste III were treated as described in Example 1. It was applied onto a chromatography column, washed at each of 3 runs with 20 mM of piperazine and 120 mM of sodium chloride at a pH of 6, and eluted with 20 mM of piperazine and 175 mM of sodium chloride at a pH of 6. The three IgM eluates were combined, and 3 l of the resulting 12 were treated as described in Example 5.

The other 9 l were irradiated in a rotary-circulation apparatus with two 20 W ultraviolet lamps at 600 rpm and a throughput of 20 l per hour at a distance of 1 cm.

Subsequent to concentration to 40 g of protein/l by ultrafiltration, half of the solution was treated with 0.05% β-propiolactone for 90 minutes at a pH of 7.2 and a temperature of 25° C.

The other half of the solution was treated for 4 hours at 25° C. with 0.3% tri-N-butyl phosphate (TNBP) and 1% Tween 80. Both batches were then treated as described in Example 5.

The two preparations were adjusted to the same protein and IgM contents. Table 5 illustrates the anticomplementary activity and the antibacterial titer.

TABLE 5

	ACA CH 50/ml	Rec. Titer *Pseudomonas aeruginosa*
Unsterilized	32	20 480
Sterilized with UV & BEL	30	10 420
Sterilized with UV, TNBP, and Tween	31	20 480

Subject to the conditions acceptable for adequate inactivation of human-pathogenic viruses, neither the anticomplementary activity nor the antibacterial action of the IgM preparation altered significantly.

The immunoglobulin preparations in accordance with the invention, which can be made available as is or optionally in the form of solutions that must be diluted before injection or freeze-dried, can also contain additional proteins (human albumin for example), sugars (glucose for example), amino acids, or appropriate monoclonal antibodies.

It will be appreciated that the instant specification and claims are set forth by way of illustration and not limitation, and that various modifications and changes may be made without departing from the spirit and scope of the present invention.

We claim:

1. A process for preparing a polyclonal chemically unmodified immunoglobulin preparation wherein at least 5% by weight of all the immunoglobulin it contains is IgM, which is low in anticomplementary activity, and which is directly administrable intravenously, from polyclonal material which is either a chemically unmodified human Cohn fraction II/III or a chemically unmodified human Cohn fraction III that contains immunoglobulins which comprises passing the polyclonal material through an anion exchange column under conditions to absorb the immunoglobulins to the column, passing through said column an eluant, wherein said eluant comprises a sodium chloride gradient with a concentration of 20 mM to 175 mM and a buffer under acidic conditions, such that high anticomplementary activity immunoglobulins remain bound to the column while low anticomplemetary immunoglobulins are selectively eluted, and collecting the eluate containing said low anticomplementary immunoglobulins.

2. The method according to claim 1, wherein the initial polyclonal material or the eluate is heated to 40° to 60° C. for 1 minute to 24 hours.

3. The method according to claim 1, wherein the initial polyclonal material or the eluate is incubated at a pH of 3.5 to 5 for 1 minute to 24 hours.

4. The method according to claim 1, wherein the anion exchanger is a polymer with a TMAE (trimethyl amino ethyl) group or a QMA (quarternary amino ethyl) group.

5. The method according to claim 1, wherein the anion exchanger is a polymer with a DEAE (diethyl amino ethyl) group.

6. The method according to claim 1, wherein the buffer comprises 20 mM of piperazine.

7. The method according to claim 1, wherein the eluant has a pH in the range of 4.7 to 6.

8. The method according to claim 1, wherein the anion exchanger is a member selected from the group consisting of

a) a copolymer of the primary monomer N-Acryloyl-2-amino-2-hydroxy-methyl-1,3-propandiol and a trimethylaminoethyl-derivative of the monomer, and

b) a natural polysaccharide with quaternary amino groups

* * * * *